

# Exploring Symbiotic Microbial Diversity in Marine Sponges

**Resource Type:** Curriculum: Laboratory

**Publication Date:** 9/12/2000

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## Abstract

The purpose of this laboratory activity is for students to examine bacterial symbiont communities harbored by marine sponges using molecular protocols. Students will learn how alternative techniques (e.g., the polymerase chain reaction (PCR)) can be used to explore microbial diversity in a novel ecosystem.

## Activity

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## INTRODUCTION

### Using PCR to Amplify 16S rDNA from Culturable and Unculturable Bacteria

Background.

#### *Microbial diversity.*

Understanding the natural world depends in part on being able to identify the species present on the planet. Compared to our knowledge of organisms such as mammals, our understanding of the microbial world is meager. It has been suggested (DeLong, 1998) that prokaryotes are the "least well understood component of the Earth's biota," with only 1 to 10% of the prokaryotes identified. Identifying prokaryotes using conventional culturing techniques does not take into account the large number of microbes that are currently unculturable. For example, *Escherichia coli* is arguably the most widely studied microbe, yet this species makes up only 0.01% of the cells found in the bacterial community of vertebrate digestive tracts (Pedros-Alio, 1993). Thus, it is probable that cultured species provide an incomplete picture of actual microbial ecosystems. Modern molecular biology provides a number of tools that permit the exploration of the tremendous diversity contained within the unculturable prokaryotic world. More specifically, the sequences of 16S rDNA genes from unculturable organisms can be obtained by PCR and compared to a database of 16S rDNA sequences. The sequences can be used to infer the phylogenetic relationships among unknown symbiotic bacteria and microbes that have already been characterized.

#### *Bacterial symbioses.*

A substantial fraction of the undescribed, undiscovered prokaryotic world may exist as symbionts living in eukaryotic hosts. Bacterial symbioses involving eukaryotic hosts are common in nature. Many bacterial symbioses support entire ecosystems. For example, deep-sea hydrothermal vent communities rely upon the chemoautotrophic capabilities of bacteria found in these environments (Cavanaugh, 1994). Other well known bacterial symbioses broaden the niches available to hosts. For instance, root nodule symbioses allow plants to grow in soils of marginal fertility (Benson and Silvester, 1993).

#### *Sponge symbioses.*

Prokaryotic symbionts are a defining characteristic of marine sponges. As with most other natural communities of bacteria, a

relatively small percentage (10%) of sponge symbionts are culturable (Santavy et al., 1990). The diverse population of symbionts can make up a large proportion of the host sponge biomass (56% in the sponge *Xestospongia* sp., (Brantley et al., 1995)). This would represent a pathological condition in most other animal taxa so it seems that the host and symbionts benefit from the association. From a medical standpoint, researchers have become extremely interested in the microbial symbionts harbored by sponges because they may be involved in the production of pharmaceutically promising natural products (e.g., anticancer drugs – see cover of *Smithsonian* Aug. 1998).

### Purpose.

The purpose of this laboratory is to examine a bacterial symbiont community harbored by a tropical marine sponge using molecular phylogenetic techniques. In this laboratory exercise, a small portion of the 16S rDNA gene will be amplified from total sponge DNA. This DNA sample will include the sponge genomic DNA and the DNA from any symbionts. An individual bacterial DNA sample (e.g., *E. coli*) will be used as a positive PCR control and H<sub>2</sub>O will be used as the negative PCR control.

### PROCEDURE

#### Materials.

DNA thermal cycler  
 PCR reaction tubes  
 1.5 ml tubes  
 PCR reaction components (10X PCR buffer, 25 mM MgCl<sub>2</sub>, 2.5 mM dNTPs, 10 mM primers, sponge/bacterial DNA, *Taq* DNA polymerase, sterile H<sub>2</sub>O)  
 Micropipettors and sterile tips  
 Disposable gloves  
 Beaker for waste/used tips  
 Agarose gel electrophoresis chambers  
 1X TBE running buffer  
 1.4% agarose  
 Loading dye  
 EtBr (1 mg/ml)  
 UV transilluminator  
 Polaroid camera and film

#### Instructor Version.

##### Lab #1: PCR of 16S rDNA.

1. Label 3 PCR reaction tubes as 1, 2, and 3.
2. Use the table below to make the PCR reaction mix cocktail in a sterile 1.5 ml microfuge tube. Use a fresh tip for each step.

Material	Final concentration	Volume per reaction	Master cocktail mix*
10X reaction buffer	1X	5 ml	15.5 ml
MgCl <sub>2</sub> (25 mM)	2.5 mM	2 ml	6.2 ml
dNTPs (2.5 mM)	200 mM	4 ml	12.4 ml
primer A (10 mM)	25 pmoles	2.5 ml	7.75 ml
primer B (10 mM)	25 pmoles	2.5 ml	7.75 ml
<i>Taq</i> polymerase (5U/ml)	2 U	0.4 ml	1.3 ml
H <sub>2</sub> O		28.6 ml	88.66 ml
Total reaction volume		50 ml	

\*Multiply each item in the volume per reaction column by 3.1. The addition of the factor 0.1 to the number of reaction tubes allows for any errors associated with pipetting small volumes of each solution.

3. After adding all reagents to the cocktail, close the tube and mix the contents.
4. Use a fresh tip to transfer 45 ml of the cocktail reaction to each of the 3 PCR reaction tubes. To each tube, add the DNA according to the table below.

Tube	Reaction cocktail	Sponge/bacterial DNA	Bacterial DNA	H <sub>2</sub> O
#1 (experimental)	45 ml	5 ml (approx. 200 ng)	-	-
#2 (positive control)	45 ml	-	5 ml	-
#3 (negative control)	45 ml	-	-	5 ml

If necessary, add an oil overlay to each sample

5. Place the reaction tubes in the DNA thermal cycler and cycle under the following conditions: 94°C for 5 minutes, then

30 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute. Allow for a final extension step of 72°C for 5 minutes.

6. Reactions can be stored at 4°C or -20°C until the next lab.

#### Lab #2: Agarose gel electrophoresis of PCR reactions.

1. Prepare gel casting tray and carefully pour agarose solution into the casting tray so that the gel covers 1/3 to 1/2 of the comb.
2. When agarose gel has set, cover gel with 1X TBE running buffer and carefully remove the comb.
3. From each of the PCR reactions (1-3) remove 15 µl of the amplification product and place in a clean microfuge tube. Add 3 µl of gel loading dye to the product and mix.
4. Carefully load the reactions into the agarose gel and electrophorese at 100 volts for 40 to 50 minutes. The bromophenol blue band should be at least halfway down the gel.
5. Turn off the power supply, remove the gel, and view the gel on the UV transilluminator.
6. Take a polaroid picture of the gel for your lab write-up.

PCR reaction 2 (bacterial DNA) should contain a single band representing the bacterial 16S rDNA. PCR reaction 3 should not have any bands (if you do have a band(s) in this lane, propose a reason for this). PCR reaction 1 should have several bands. One of the bands represents the sponge's 16S gene (~550-600 bp) from mtDNA. The other amplification products represent 16S rDNAs from unknown microbial organisms that are living in a symbiotic relationship with the sponge. If you needed to determine the identity of each of the bands, propose an experiment that would allow you to characterize each of the bands.

#### Safety Issues.

Gloves and laboratory coats should be worn at all times when performing these exercises. The chemical ethidium bromide is a mutagen and suspected carcinogen, use care when handling the agarose gel and running buffer. The agarose gel and gel running buffer should be disposed of in marked containers in the laboratory. When viewing the agarose gel under UV light always wear eye protection or use a UV shield, as UV light can damage the retina.

Some individuals are allergic to marine sponges. Take care to have students either wear gloves when handling them or wash hands immediately after handling.

#### ASSESSMENT and OUTCOMES

Suggestions for Assessment.

We usually have students hand in a formal lab write-up for this activity. They are asked to include a discussion of how they might specifically identify the bacterial species represented in the amplified 16S rDNA fragments (e.g., clone and sequence the bands and compare to the Genebank bacterial database).

#### Problems and Caveats.

The preparation time for lab 1 includes time needed to prepare sponge/bacterial DNA. DNA isolation can be done any time before the lab, as DNA can be safely stored for many months at -20°C. It will take approximately 1 to 2 hours to prepare the DNA (see appendix). The PCR set-up for lab 1 takes about 2 hours. To simplify the lab procedure, the instructor can make the master mix PCR cocktail for the students ahead of time. The laboratory set-up for lab 2 is also about 2 hours. This is provided that the institution where this is being performed has all of the standard equipment for PCR and gel electrophoresis and that the instructor has some background in these techniques. It will be necessary for the instructor to include a discussion about PCR and gel electrophoresis if these topics have not already been covered in class. Because there are so many good sources on these topics (e.g., Sambrook et al., 1989), we did not include detailed explanations of these techniques here.

#### SUPPLEMENTARY MATERIALS

##### Possible Modifications.

1. The instructor can set up a visual display of the sponge from which the DNA for the PCR is extracted. Examples from nature of other microbe-host interactions, such as the *Rhizobium*-clover root nodule symbiosis, the *Frankia*-actinorhizal root nodule symbiosis, or the termite hindgut-microbe symbiosis, also may be presented.
2. The instructor can have students use an inoculum of homogenized sponge tissue to cultivate and phenotypically characterize culturable microbial communities on marine media.
3. The laboratory exercise we propose will demonstrate to students the importance of molecular techniques in the investigation of unculturable microorganisms. Since 16S rDNA cloning, sequencing, and analysis is a standard protocol after PCR to characterize unculturable microbial populations, our laboratory naturally ties in to the computer exercise "Identification of Bacterial Unknowns by rRNA Sequence Similarity" written by Richard Røller (found in the ASM Education Instructional Library). This exercise introduces students to database analysis of 16S rDNA sequences derived from unknown microorganisms. Fifty 16S rDNA sequences collected from sponge bacterial symbionts have recently been deposited in Genebank and these sequences could also be used for this analysis. The proposed exercise and the "Identification of Bacterial Unknowns by rRNA Sequence Similarity" exercise are complimentary and provide students with a complete picture of how molecular techniques and database analysis can provide new insight into uncultivated microorganisms of complex ecosystems.

#### References.

1. **Benson, D. R. and W. B. Silvester.** 1993. Biology of *Frankia* strains, Actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* **57**:293-319.
2. **Bloom, M. V., G. A. Freyer, and D. A. Micklos.** 1996. Laboratory DNA science: an introduction to recombinant DNA techniques and methods of genome analysis. The Benjamin/Cummings Publishing Company Inc., Menlo Park, Calif.
3. **Brantley, S. E., T. F. Molinski, C. M. Preston, and E. F. DeLong.** 1995. Brominated acetylenic fatty acids from *Xestospongia* sp., a marine sponge-bacteria association. *Tetrahedron* **51**:7667-7672.
4. **Cavanaugh, C. M.** 1994. Microbial symbiosis - patterns of diversity in the marine environment. *American Zoologist*

34: 79-89.

5. DeLong, E. 1998. Archaeal means and extremes. *Science* **280**:542-543.
6. Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction. p. 205-247. *In* D. M. Hillis, C. Moritz, and B. K. Mabel (ed.). *Molecular systematics*, 2nd edition. Sinauer Associates Inc., Sunderland, Mass.
7. Sambrook, J. E., et al. 1989. *Molecular cloning—a laboratory manual*. Cold Spring Harbor, Cold Spring Harbor Press, Woods Hole, Mass.
8. Santavy, D. L., P. Willenz, and R. R. Colwell. 1990. Phenotypic study of bacteria associated with the Caribbean sclerosponge *Ceratoporella nicholsoni*. *Appl. Environ. Microbiol.* **56**:1750-1762.

## Appendices.

### Sponge samples.

Shipment of sponges is at the discretion of the supply company, but typically takes 2 to 4 weeks. In the table below, we have listed some of the species that are typically available. Small fragments of live sponge tissue can be excised and frozen at  $-80^{\circ}\text{C}$  for use in subsequent labs. It is not necessary to keep sponges in marine tanks if tissue is excised and frozen upon arrival.

Biological supply company	Species	Characteristics of interest
Carolina Biological Supply 2700 York Road Burlington, NC 27215 (800) 227-1150	<i>Haliclona rubens</i> <i>Chondrilla nucula</i>	Both of these sponges are common Caribbean sponges. <i>C. nucula</i> harbors dense populations of cyanobacteria.
Gulf Marine Specimens P.O. Box 237 Panacea, FL 32346 (850) 984-5297	Call for available species.	
Animal Supply House Marine Biological Laboratory Woods Hole, MA 02543 (617) 548-3705 x325	<i>Microciona prolifera</i> <i>Cliona celata</i> <i>Haliclona</i> sp. <i>Halichondria</i> sp.	Each of these species is found in temperate waters of the northeast U.S.
Pacific Bio-Marine Laboratories, Inc. P.O. Box 536 Venice, CA 90291 (323) 822-5757	Call for available species.	
Marinus, Inc. 1500 Pier C Street Long Beach, CA 90813 (310) 435-6522	Call for available species.	

\* Freshwater sponges can be ordered from many supply companies but these species *do not* appear to harbor bacterial symbionts.

### DNA isolation.

We usually prepare the bacterial/sponge DNA before the lab using the QIAGEN (Qiagen, Inc., Valencia, Calif.) tissue extraction kit. We use the protocol specifically designed for bacteria (QIAamp tissue kit protocol D). This produces large quantities of high quality DNA that can be used directly in the PCR. Additionally, DNA from a bacterial species grown in a monoculture (e.g., *E. coli*.) should be prepared for use as a positive control for the PCR reactions. We usually use approximately 200 ng of DNA per reaction.

### PCR set-up.

We have successfully used the universal PCR primers 16Sar (5'CGCCTGTTTATCAAAAACAT3') and 16Sbr (5'CCGGTCTGAACTCAGATCACGT3') which are complementary to conserved regions of the 16S rRNA genes. Information about these primers can be obtained from Palumbi (1996). We have also amplified 16S genes using the universal bacterial primers fD1 and rD1 (Weisburg et al., 1995). Each group of students will need 10 ml of a 10 mM stock of each primer. For PCR reactions, we use reagents from PE Applied Biosystems (Foster City, Calif.). Amplitaq DNA polymerase (catalog #N808-0161) is supplied with 10X reaction buffer,  $\text{MgCl}_2$  (25mM) and dNTPs (10 mM). We make a 2.5 mM dNTP stock.

### Agarose gel electrophoresis.

Standard procedures for agarose gel electrophoresis can be obtained in Sambrook et al (1989). Prepare enough 1.4% agarose gel in 1X TBE buffer for each group. Before students pour the gels, add about 7.5 ml of a 1 mg/ml ethidium bromide (EtBr) solution to 100 ml of a cooled 1.4% agarose gel solution ( $60^{\circ}\text{C}$ ). Use proper procedures for handling and disposing of EtBr. We use the S&S Extractor® Ethidium Bromide Waste Reduction System from Schleicher & Schuell (item #448031) to decontaminate the running buffer. The gels should be stored in a chemical waste container and disposed of according to state and federal procedures for hazardous chemical wastes. Alternatively, the gels can be decontaminated using protocols described in "Laboratory DNA Science" by Bloom, Freyer, and Micklos (1996). For viewing the agarose gel using UV illumination, use a UV transilluminator with a shield if available. Alternatively, have students wear protective eye wear or face shields.

Results: It should be noted that sponges are filter-feeders and some of the PCR products may come from recently consumed bacteria. Thus, in situ hybridization is one of the best ways to localize particular symbionts. Students may be able to propose additional techniques that would help rule out the possibility of amplifying nonsymbiotic bacteria.

### Recipes for Media.

The recipe for TBE running buffer can be found in Sambrook et al. (1989).