

Feb 14, 2024

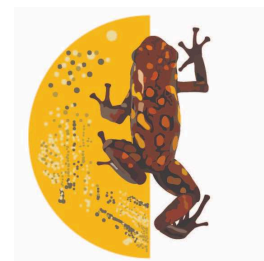
Version 1

Isolation of microbes from the skin of terrestrial frogs V.1

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We use this protocol and it's working

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Abstract

The microbes on amphibian skin are critical for host health. Beneficial skin microbes regulate the growth of other microbes and influence many important host physiological processes. This protocol details how to isolate microbes from the skin of terrestrial frogs, using poison frogs as an example. This assay is useful in research laboratories, undergraduate teaching laboratories, and in field sampling of wildlife. Completion of the protocol should result in the isolation of several bacteria or fungi that can be identified using DNA barcoding and used for further experimentation in microbial ecology.

Attachments



[Swabbing and Plating...](#)

418.7MB

Image Attribution

Image art by Lauren A. O'Connell

Guidelines

This protocol serves as a starting point, but swabbing approaches may need to be adjusted depending on the size of the animal.

Materials

Preparation of media and plates for microbial cultures

MilliQ or DI water

Glass culture media bottle (Catalog #CLS13951L)

Agar (Fisher Scientific Catalog #BP97445)

NaCl (Sigma-Adrich Catalog #S5886-500G)

Tryptone (Fisher Scientific Catalog #BP1421-500)

100 mm X 15 mm Petri Dishes (Fisher Scientific Catalog #FB0875713)

Parafilm M Sealing Film (Millipore Sigma Catalog #HS234526B)

Disposable Inoculating Loops and Needles (Fisher Scientific Catalog# 22-363-605)

Swabbing frogs

Sterile Dry Applicator Collection Device (Weber Scientific Catalog # 3059-46)

PCR amplification

OneTaq Hot Start Quick-Load 2X Master Mix (New England Biolabs Catalog # M0488)

16S Primers: 10uM Forward: 27F (5'-AGA GTT TGA TCM TGG CTC AG-3')

10uM Reverse: 1492R (5'-TAC GGY TAC CTT GTT AYG ACT T-3')


ITS Primers: 10uM Forward: ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')

10uM Reverse: glITS7 (5'-GTG ART ATC GAR TCT TTG-3)

Nuclease free water (e.g. New England Biolabs Catalog #B1500L)

Troubleshooting

Safety warnings

 This protocol includes use of an open flame, which is a fire hazard. Take care to make sure the area is clear of clutter and fire mitigation tools are available.

Ethics statement

Experiments involving animals must have prior approval from an Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.











Before start


This work should be done in a sterile environment, if possible. Perform all work on a clean and disinfected benchtop with a Bunsen burning flame on. Always work close to the flame, as the flame produces an upward current of the air that keeps microbes in the air from contaminating your plate.



Prepare plates for isolation of microbes

- 1 Prepare a number of rich, non-specific media plates for growing as many strains as possible. One or all types could be used, or alternative media could be used when appropriate. All media are autoclaved at  121 °C to sterility and specific times depend upon the volume of media you make.
- 2 **Prepare 1% Tryptone mixture for pouring 500mL or 25 100mm x 15mm plates.** 10m
 - 2.1 Wear gloves disinfected with 70% ethanol throughout the procedure.
 - 2.2 Place an autoclaved magnetic stir bar into a sterile 1L glass culture media bottle.
 - 2.3 Add  250 mL of MilliQ water into the bottle. Set the bottle on a heat plate at room temperature, ensuring the stir bar is active.
 - 2.4 Incorporate dry Ingredients to the stirring solution:
 -  8 g of Agar
 -  4 g of Sodium chloride (NaCl)
 -  5 g of Tryptone
 - 2.5 Add an additional  250 mL of MilliQ water to the mixture. Allow all ingredients to mix thoroughly.
- 3 **Autoclave the media to sterilize the contents.** 2h
 - 3.1 To autoclave the mixture, use the Liquid cycle at  121 °C for  00:40:00 . Place the bottle in a suitable container and fill it with water up to the level of the mix inside the bottle. 40m




3.2 When the cycle is finished, open the autoclave door about one inch for  00:10:00 to let the steam out. 10m

3.3 Discard the water from secondary container and place the bottle with the mixture on the heat plate.



3.4 Visually inspect the mixture to ensure homogeneity, verifying that it has a yellow appearance.
If the mixture displays distinct clear and yellow phases or streaks, mix again on the heat plate and repeat the liquid cycle.

3.5 Allow the media to cool to a suitable handling temperature.




4 **Pour the autoclaved media into plates.** 30m


4.1 Sterilize the surface thoroughly with 70% ethanol to ensure aseptic conditions. Light a Bunsen burner and allow enough space to work around it when pouring the plates. Only open the lid of the media bottle close to the flame. 

4.2 Set five plates close to the burner and start pouring the mixture until the bottom surface of the plate is covered. To avoid contamination, lift the cover of every plate, pour the mixture, and immediately cover the plate again.

4.3 Stack the plates to help clear condensation in the lid. Allow  120:00:00 to verify that there is no contamination in the plates before storing or using. Store at  4 °C until ready for use. 5d

5 **Preparation of 1L of solid media with Defibrinated Sheep's Blood** 2h




-  40 g Tryptic Soy Agar (BD 236950)
-  50 mL Sheep Blood (defibrinated)
-  950 mL MilliQ or DI Water


Autoclave water and Tryptic Soy Agar, let cool to ~  47 °C , add sheep blood, then dispense into plates.



6 Preparation of 1L of Tryptic Soy Broth with Defibrinated Sheep's Blood

2h

-  30 g Tryptic Soy Broth (BD 211825)
-  50 mL defibrinated Sheep Blood
-  950 mL MilliQ or DI Water

Autoclave water and Tryptic Soy Broth, let cool to ~  47 °C , then add sheep blood.

7 Other media used for characterization:

2h

R2A

Liquid and solid media are made following manufacturer's instructions. (BD 218263)

LB

Liquid and solid media are made following manufacturer's instructions. (BD 244620)

YPD





Liquid and solid media are made following manufacturer's instructions. (BD 242720)

YPD is especially useful for culturing fungi.

Prepare liquid media for growing isolated microbes

8 Preparation of 1L of Tryptone 1% Liquid Medium for Microbial Cultures

2h

- 8.1 Mix together  4 g of Sodium chloride (NaCl) and  5 g of Tryptone in  900 mL water a large beaker Stir using a magnetic stir bar.
- 8.2 When ingredients are incorporated into the water, add additional water to a final volume of 1L.
- 8.3 Transfer the mixture into small glass bottles, aiming for approximately 12 bottles per liter and ensuring they are not filled beyond half capacity. Utilize a pipet to prevent any spillage onto the bottle sides.
- 8.4 Secure the bottle lids loosely and place them in an autoclave for a minimum of  00:30:00 within a secondary containment, with 2 centimeters of water.

30m



- 8.5 Once autoclaved, tighten the bottle lids and seal the opening with tape, indicating the date.
Tear the tape when the bottles are opened.
- 9 Liquid media is used for making glycerol stocks, as outlined in Step 21 below.

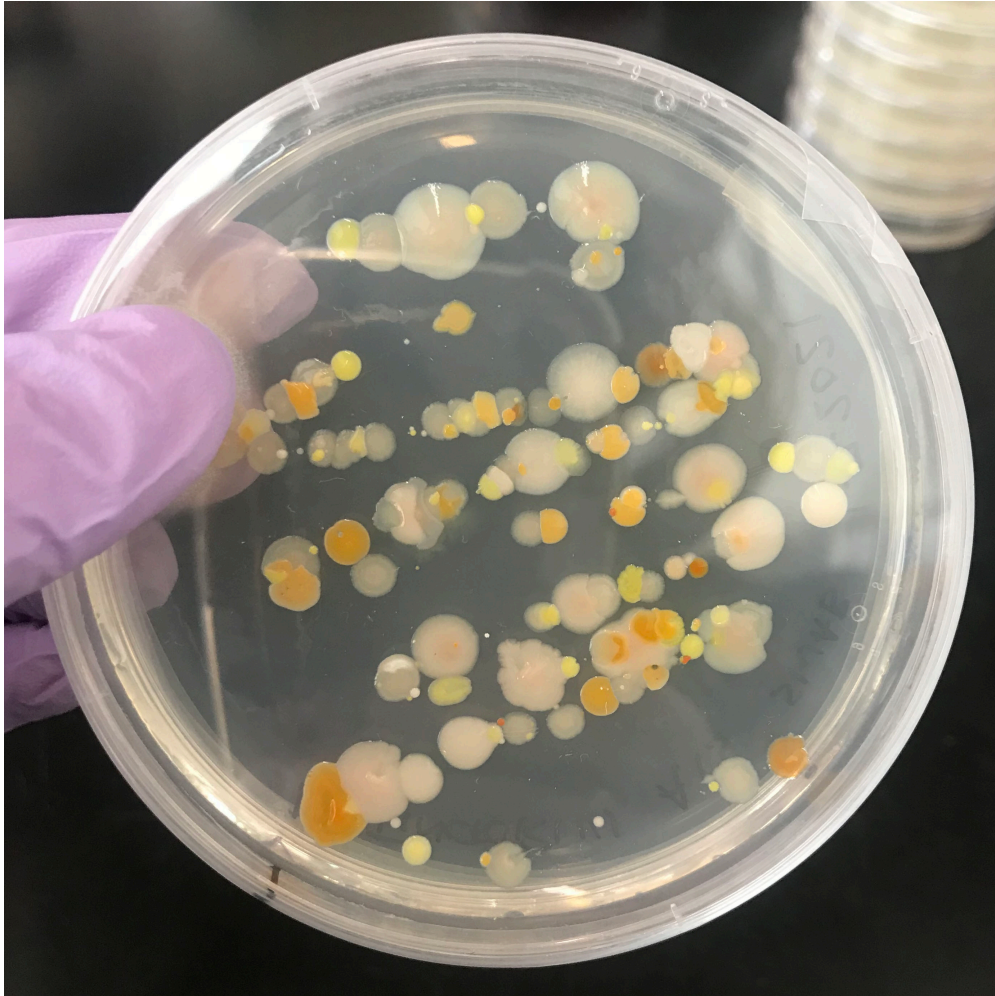
Transfer of skin microbes from frog to plate.

- 10 Maintain a sterile environment throughout the work. Keep gloves on at all times and disinfect them with 70% ethanol. Allow ethanol to evaporate before handling frogs and change gloves before handling the next individual.
- 11 Open a sterile cotton applicator and wet the applicator with sterile water.
- 12 Catch the frog with a fresh plastic bag. Hold one or both legs between thumb and index finger, then open the bag and expose the frog. Rinse the frog with sterile distilled water using an autoclaved squirt bottle. This process minimizes transient bacteria or soil particles on the skin. 5m
- 13 Swab the frog 10 times on each side of your interest (up and down the back is once), e.g. on the dorsal side, the legs, on either side and on the ventral side, including the fingers. Different body parts of the frog will have different microbes. For example, chytrid fungi will be found primarily on the ventral side and between the digits. 1m



Swabbing of the diablito frog (*Oophaga sylvatica*)

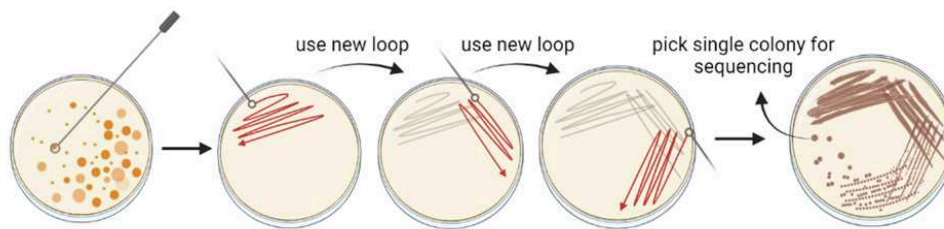
- 14 Transfer microbes from the swab to a growth plate. 3m
- 14.1 Open the plate. Hold the swab in one hand and pick the plate up with the other hand. Streak the cotton applicator across the plate. Turn the plate and repeat the streaking a second time.
- 14.2 Close the plate, turn it around and label the outer rim of the bottom carefully with experimenter initials, plate type (ex. 1% Tryptone or YPD), the date and the name of the frog species that was swabbed.
- 15 Close the plate with parafilm and incubate it at the desired temperature (e.g. the temperature of the frog's natural environment). Visible colonies should appear after an overnight incubation.



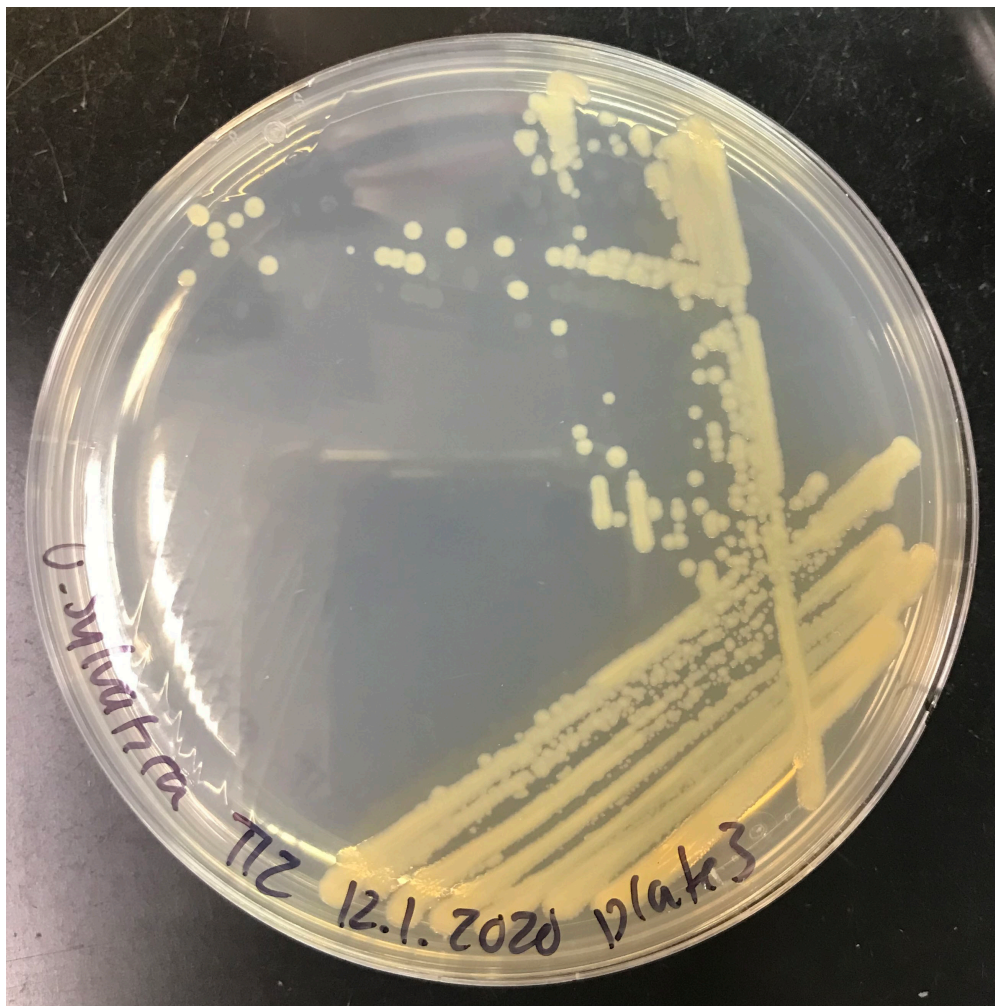
Colonies growing on plate after frog swabbing.

Isolation of microbe strains

- 16 When colonies are large enough to be picked, touch a sterile loop or needle tool to an individual colony, and streak out onto a new plate of the same media type. Label plate and wrap with parafilm. Incubate overnight or until new colonies are visible.

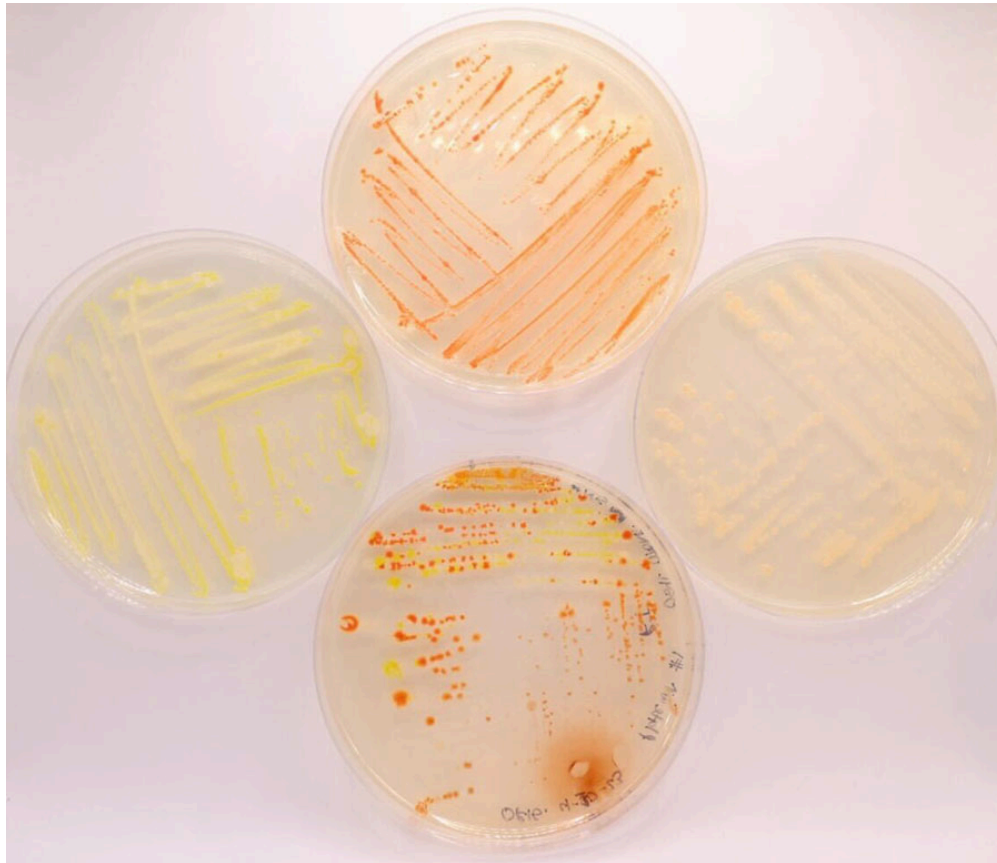


Isolation streaking. Created with [BioRender.com](https://www.biorender.com/).





Streak of microbial isolate from the diablito frog (*Oophaga sylvatica*).




- 17 Again select single colonies and streak out onto new plates.













Several plates of microbial isolates from the diablito frog (*Oophaga sylvatica*).

- 18 Repeat Step 17 for a third isolation step.
- 19 To identify colonies, scrape half of the colony selected for strain identification off the plate using a pipette tip. Use half to grow the microbe overnight (see Step 20) and the other half place the pipette tip into  10 μL nuclease free water (See PCR below).
- 20 Grow the strain overnight in liquid media that corresponds to the isolation media. 12h
- 21 Combine equal amounts of 50% glycerol solution with the overnight cultures. Store at  $-80\text{ }^{\circ}\text{C}$ for future use. Making several stocks is recommended. 10m
- 21.1 Make glycerol:
 1. Combine 1:1 glycerol and milliQ water and gently mix to combine



2. Vacuum filter with 0.22 μm filter
3. Label two cryotubes per isolate
4. In each tube, add:
 -  500 μL of glycerol
 -  500 μL of liquid culture
5. Store in  $-80\text{ }^{\circ}\text{C}$

PCR amplification of microbial DNA

- 22 Dilute the strains in water by placing half of a single colony into  10 μL of sterile water using a pipette tip. Use  1 μL of diluted strains as input for PCR.
- 23 Set up a PCR reaction amplify regions of the microbial genome for sequencing.
 - 23.1 To identify bacteria, set up a PCR reaction to amplify the full length 16S gene. Each reaction should contain:
 -  1 μL diluted strain
 -  1 μL forward primer (27F)
 -  1 μL reverse primer (1492R)
 -  12.5 μL OneTaq or other polymerase suited for amplification of long pieces of DNA
 -  9 μL nuclease free water
 - 23.2 To identify fungi, set up a PCR reaction to amplify a portion of the ITS gene. Each reaction should contain:
 -  1 μL diluted strain
 -  1 μL forward primer (ITS4)
 -  1 μL reverse primer (gITS7)



- 12.5 μL OneTaq or other polymerase suited for amplification of long pieces of DNA
- 9 μL nuclease free water

24 Place the PCR reactions in a thermal cycler and run the amplification protocol.

24.1 For the bacterial samples, run a PCR with the following parameters:

4h

1 cycle of 00:00:30 at 94 °C

followed by

30 cycles of 00:00:15 at 94 °C , 00:00:30 at 55 °C and

00:02:00 at 65 °C ,

then a final extension period of 00:05:00 at 65 °C .

24.2 For fungal samples, run a PCR with the following parameters:

4h

1 cycle of 00:00:30 at 94 °C followed by

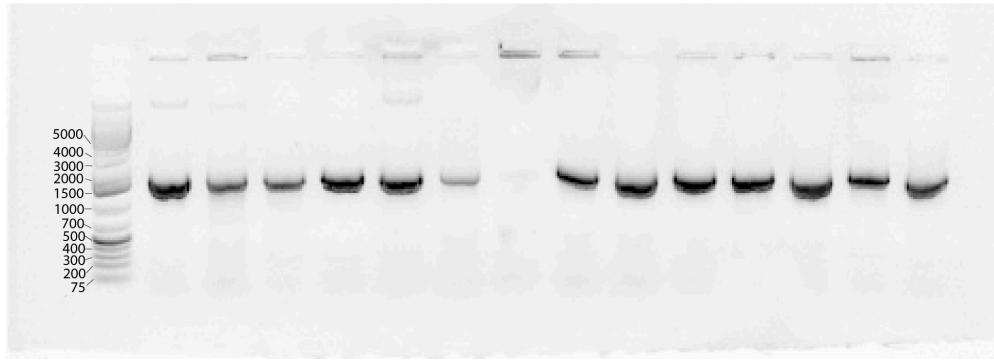
30 cycles of 00:00:15 at 94 °C , 00:00:30 at 55 °C and

00:02:00 at 68 °C ,

then a final extension period of 00:00:15 at 68 °C .

25 Confirm successful amplification with gel electrophoresis. If amplification is unsuccessful, the strain may be a fungus or a Gram-positive bacteria, which will not lyse easily in water.

1h



Example of gel image from 16S PCR products. Most reactions resulted in successful amplification, except for the center lane. DNA ladder is labeled with number of base pairs for each band.

- 25.1 If no amplification occurred, grow up new colonies from the glycerol stock and extract DNA using the Qiagen Blood and Tissue Kit adapted for samples from Gram positive bacteria. 1h
- 26 Purify the PCR products for sequencing using a PCR purification kit. Suggested kit: E.Z.N.A Cycle Pure Kit from Omega. 1h
- 27 Submit samples for Sanger sequencing. Provide primers for sequencing in both directions (forward and reverse) separately.

Identification of microbe strains

- 28 Align forward and reverse reads and compare to reference databases with BLAST on the NCBI platform.



Protocol references

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