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Protocol for the generation of axenic/bacteria-depleted Symbiodiniaceae cultures

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We use this protocol and it's working

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Abstract

This protocol produces bacteria-depleted (“axenic”) Symbiodiniaceae cultures. The depletion level achieved using the following methods is comparable to what was previously described as an “axenic” culture (Bieri et al., 2016; Xiang et al., 2013). The present protocol was applied to 10 Symbiodiniaceae strains and proved successful in generating bacteria-depleted cultures after its application, for an extended period of time (up to 4 weeks), characterized by both culture-dependent and -independent techniques. Tested cultures showed normal growth and photosynthetic efficiency after recovery from the treatment. Variations of the protocol were developed to increase the success of bacterial depletion for different microalgal strains and to make it applicable to algal strains that do not grow well on solid media. Treatments can also be performed in 24-well plates in order to facilitate the measurement of F_v/F_mthrough the plate bottom using a fiber optic cable, in order to track photophysiology. This protocol was developed in parallel with a recently published method (Costa et al., 2019) to generate bacteria-depleted Aiptasia CC7 polyps, in order to have a toolbox at disposal to disassemble the different compartments of an anthozoan metaorganism for functional testing: the bacterial microbiome, the microalgal symbionts, and the anthozoan host animal. All steps of culture manipulation in this protocol must be done under a sterile environment, using common sterile work practices!

Attachments

PDF

Protocol for the gen...

231KB

Guidelines

All steps of culture manipulation in this protocol must be done under a sterile environment, using common sterile work practices!

Materials

Reagents

For culture maintenance:

- Guillard's (F/2) Marine Water Enrichment Solution 50x (Sigma G0154)
- Agar for general bacteriological purposes (we used Difco™ Noble Agar 214230 for extra clarity)
- Natural autoclaved seawater or Artificial seawater (ASW):
  - 420 mM Sodium chloride – CAS: 7647-14-5
  - 10.5 mM Calcium chloride dihydrate – CAS: 10035-04-8
  - 840 µM Potassium bromide – CAS: 7758-02-3
  - 71.5 µM Sodium fluoride – CAS: 7681-49-4
  - 9.4 mM Potassium chloride – CAS: 7447-40-7
  - 485.2 µM Boric acid – CAS: 10043-35-3
  - 28.8 mM Sodium sulfate – CAS: 7757-82-6
  - 2.4 mM Sodium bicarbonate – CAS: 144-55-8
  - 63.8 µM Strontium chloride hexahydrate – CAS: 10025-70-4
  - 58.3 mM Magnesium chloride hexahydrate – CAS: 7791-18-6
  - For pH adjustment: Sodium hydroxide – CAS: 1310-73-2

For Antibiotic treatment:

- Antibiotic 'cocktail'/solution, ABS:
  - 50 µg/ml Rifampicin – CAS: 13292-46-1; Stock solution as 50 mg/ml in 100% DMSO
  - 30 µg/ml Nalidixic acid – CAS: 389-08-2; Stock solution as 50 mg/ml in MilliQ H_2O with pH adjusted to 11 using NaOH
  - 50 µg/ml Carbenicillin – CAS: 4800-94-6; Stock solution as 100 mg/ml in MilliQ H_2O
  - 25 µg/ml Nystatin – CAS: 1400-61-9; Stock solution as 5 mg/ml in 100% DMSO
  - 50 µg/ml Erythromycin – CAS: 114-07-8; Stock solution as 10 mg/ml in Absolute ethanol

- For Antibiotic agar plates (ABS2):
  - 100 µg/ml Ampicillin Sodium Salt – CAS: 69-52-3; Stock solution as 100 mg/ml in MilliQ H_2O
  - 50 µg/ml Streptomycin sulfate salt – CAS: 3810-74-0; Stock solution as 50 mg/ml in MilliQ H_2O
  - 200 µg/ml Gentamicin sulphate – CAS: 1405-41-0; Stock solution as 50 mg/ml in MilliQ H_2O

For bacterial contamination assessment:

- Difco™ Marine Agar 2216 and any other rich medium
- Qiagen DNeasy Blood & Tissue Kit or any Bacterial DNA extraction method

- Taq-polymerase or any PCR-ready mix (we use Qiagen Multiplex PCR kit Cat No: 206145)
- 16S rRNA gene primers (we use universal primer pairs 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3', or 67F 5′-CAGGCCTAACACATGCAAGTC-3' and 1542R 5′-AAGGAGGTGATCCAGCCGCA-3'). The latter pair allows a better separation of bacterial and eukaryotic amplicons on agarose gel (Galkiewicz and Kellogg, 2008)

Consumables

- T25 cell culture flasks, vented caps or glass test tubes with metallic caps
- Sterile 24-well tissue culture plates
- Sterile plastic 100 × 15mm Petri dishes
- 1.5 ml reaction tubes
- 0.2 ml PCR tubes
- 0.2 µm pore size filter with disposable bottles (we use Corning bottle-top vacuum filter system)
- Sterile serological pipettes, 5, 10, 25 ml
- Sterile 15 ml polypropylene tubes

Equipment

- Autoclave
- Biosafety cabinet
- Inverted microscope with fluorescent module and a chlorophyll detection filter/cube
- Incubator with controlled light and temperature settings
- Thermocycler
- Electrophoresis apparatus
- PAM fluorometer (optional)

Biological material

- Clonal Symbiodiniaceae strains, isolated from animal tissue, sediment or water.

Culture Maintenance

1 Prepare artificial seawater (ASW) by mixing reagents in MilliQ water and adjusting the pH of the final volume to 8.0–8.2. Filter through 0.22 µm pore size filter. Alternatively, autoclave natural seawater and filter through 0.22 µm pore size filter (NSW).

2 Prepare F/2 medium by diluting it to 1x in ASW or NSW in a biosafety cabinet and filter again through0.22 µm pore size filter. Store the prepared F/2 medium at 4 °C and let it come to room temperature before adding to cultures.

3 Split algal cultures every 2 weeks by transferring 1 part of culture to 9 parts fresh F/2 medium.

Antibiotic Treatment

4 Prepare the antibiotic F/2 media solution (F/2 ABS):

4.1 Mix 50 µg/ml carbenicillin, 50 µg/ml erythromycin, 50 µg/ml rifampicin, 30 µg/ml nalidixic acid and 25 µg/ml nystatin with 1x F/2 media (in ASW) and sterile filter the final solution through 0.22 µm pore size filters.

> Note
>
> Cover the bottle with aluminum foil to protect from light. Alternatively, it is possible to filter the stock solutions of antibiotics and 1x F/2 media separately and freshly prepare the working ABS.

5 Prepare the antibiotic F/2 media (F/2 ABS2) agar plates:

5.1 Add the recommended manufacturer's agar quantity to a defined volume of 1x F/2 medium.

5.2 Dissolve the agar and autoclave the mixture for 20 min at 121 °C.

5.3 After autoclaving, in a biosafety cabinet, allow the agar mixture to cool down to ~60 °C before adding 50 µg/ml Streptomycin, 100 µg/ml Ampicillin and 200 µg/ml of Gentamycin and mix.

5.4 Pour the agar in the plates and allow to solidify. Close the plates and store in a sealed bag at 4 ºC until use.

6 Inoculate 10 ml of liquid F/2 medium and incubate in a 12 h light: 12 h dark incubator (35–150 µmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation) at 25 °C or at the optimal temperature of the Symbiodiniaceae strain.

7 Count the cells with a Neubauer or Nageotte chamber in the microscope, and once the culture reaches at least 10^5–10^6cells/ml, transfer 1 ml of the cell suspension to a 1.5 ml reaction tube.

8 Spin the cell suspension down at low speed (5 min, 800 x g) and rinse the pellet twice with ASW.

Note

Centrifugation at low speeds decreases the pelleting of bacteria (in comparison to microalgal cells), reducing the bacterial cargo of the initial inoculum.

Note

To check for initial bacterial load and to assess bacterial load throughout the protocol, collect aliquots as described in step 16 and compare all samples during step 17.

9 Resuspend the pellet in 1 ml of F/2 and inoculate in 9 ml of F/2 ABS.

Note

Each antibiotic targets a different mechanism of bacterial replication. Thus, the cocktail combines different bacteriostatic and bactericidal mechanisms. Nystatin targets fungal contamination. The antibiotic solution can be kept at 4 °C protected from light (but no longer than 2 weeks).

10 Incubate at the same light and temperature conditions for 7 days.

Note

Check the cultures with an epifluorescence inverted microscope with a filter/cube that detects chlorophyll. Symbiodiniaceae will lose photosynthetic efficiency (loss of red fluorescence), but will survive. Measurements of maximum quantum yield by PAM fluorometry also showed a strong effect of this cocktail on the photophysiology of cells but quantum yield recovered during steps 12, 13.

11 After 7 days of treatment, collect 1 ml of the cell suspension and repeat step 8.

12 Resuspend the rinsed cells in 10 ml of F/2 to recover and incubate as before.

13 Once the culture reaches at least 10^5–10^6 cells/ml, take 1 ml and repeat step 8.

14 Resuspend the pellet in 1 ml of F/2 and spread 50–100 µl in F/2 ABS2 agar plates. Wrap with parafilm and incubate at optimal growth temperature and light cycle until colonies form.

Note

This can take 1-3 weeks depending on the strain.

15 Pick one colony from the previous plate, resuspend it in 100 µl of F/2 and repeat step 14.

16 Pick a colony and inoculate 10 ml of F/2 medium. Let it grow until it reaches at least 10^5–10^6 cells/ml.

17 To check for depletion, remove 1 ml of culture and plate 200 µl of the supernatant in Marine Agar or Tryptic Soy Agar. Do the same thing with the original cultures as a positive control.

18 If the plates show no growth after 5–7 days of incubation (but extended incubation (>14 days) is advised), perform one more split (same way, 1:9) of the Symbiodiniaceae cultures.

19 Once they reach the desired concentration, collect 1 ml of the suspension, spin it down at high speed (≥ 14 000 x g for 5-15 min), discard the supernatant and perform DNA

extraction on the pellet.

Note

For quicker assessment, pellets were resuspended in TE buffer to algal cell concentration of 200.000 cells/ml based on the cell counts. 20 µl of these normalized suspensions were then boiled for 25 min at 100 °C. The lysate can be used directly as template in step 20. Regular DNA extraction methods can be used instead.

20 Perform a PCR of the 16S rRNA gene using 10 ng of DNA template. If using preparation methods where absolute DNA quantification is not recommended (See Note in step 19), use algal cell numbers as abasis to normalize across tested conditions. Absence of amplification or amplification comparable to the negative PCR control in an electrophoresis indicates successful treatment.

Note

Alternatively, this profile was used (primer pair 27F-1492R): 94°C for 3 min, followed by 35 cycles of each: 1 min at 94°C, 30 s at 55°C, and 90 s at 72°C. A final extension step was set at 72°C for 10 min.

Note

The PCR thermal profiles used were as follows (primer paris 27F-1492R and 67F-1542R):95°C for 15 min, followed by 30 cycles of each: 30 s at 95°C, 90 s at 55°C, and 90 s at 72°C. A final extension step was set at 72°C for 10 min

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	Culture	Species or ITS2 type	Culture collection ID	Strain Origin	Previously reported as "axenic"?	Observations	Reference
	370	*Symbiodinium microadriaticum*	CCMP2467	NCMA	No	-	LaJeunesse, 2001
	-	*Symbiodinium linucheae*	SSA01	Pringle Lab	Yes	-	Bieri et al., 2016
	-	*Breviolum minutum*	SSB01	Pringle Lab	Yes	-	Xiang et al., 2013
	74	*Breviolum minutum*	-	Warner Lab	No	-	LaJeunesse, 2001

	89	A2	-	Warner Lab	No	Strain does not grow well on solid medium	LaJeunesse, 2001
	146	*Breviolum pseudominutum*	CCMP3450	Warner Lab	No	146 & RT146 are cultures of the same strain; strain did not grow well on solid medium	Parkinson et al., 2015
	RT 146	*Breviolum pseudominutum*	CCMP3450	LaJeunesse Lab	No	146 & RT146 are cultures of the same strain; strain did not grow well on solid medium	Parkinson et al., 2015
	351	*Breviolum minutum*	-	Warner Lab	No	-	Parkinson et al., 2015
	Mf	A2	-	Warner Lab	No	Strain did not grow well on solid medium	Steinke et al., 2011
	Mac702	B2	-	Warner Lab	No	-	Santos et al., 2001

Table 1. Symbiodiniaceae strains tested for which bacterial depletion works

Variations to the protocol outlined above

22 Variation 1 - To increase the success of the protocol by increasing the stringency of the treatment, the first antibiotics treatment (steps 6-10) can be repeated before moving to step 14, without compromising viability of algal cultures. For instance, strains 89 and RT146 still yielded colonies of *Stappia indica* and *Muricauda aquimarina* | *Paracoccus homiensis*, after one round of antibiotics treatment. By contrast, no bacterial growth was detected on marine agar plates (steps 16 to 18) when steps 9-13 were performed again, i.e. twice.

23 Variation 2 - The second antibiotics cocktail (ABS2) can also be applied in liquid F/2 medium, which can be an advantage or might even be necessary when algal strains do not grow well on solid medium. From the tested strains, 89, 146, RT146 and Mf do not grow well on solid medium and depletion of bacteria was only achieved by applying the ABS2 treatment in liquid medium. However, sterility tests after several weeks following the protocol suggest that colony picking from solid medium increases the chance of gaining axenic cultures or at least extends the duration during which cultures can be considered bacteria-depleted. This effect might be due to the physical exclusion of bacterial contamination when a single isolated algal colony is picked multiple times in

solid media, versus a suspension culture split, which always carries part of the previous culture.

References

24 Bieri, T., Onishi, M., Xiang, T., Grossman, A. R., and Pringle, J. R. (2016). Relative contributions of various cellular mechanisms to loss of algae during cnidarian bleaching. PLoS One 11, 1–24.

Rúben M Costa, Anny Cárdenas, Christian Voolstra (2019). Protocol for bacterial depletion of Aiptasia anemones - Towards the generation of gnotobiotic/germ-free cnidarian host animals. protocols.io**dx.doi.org/10.17504/protocols.io.7mrhk56**

Galkiewicz, J. P., and Kellogg, C. A. (2008). Cross-kingdom amplification using Bacteria-specific primers: Complications for studies of coral microbial ecology. Appl. Environ. Microbiol. 74, 7828–7831.

LaJeunesse, T. C. (2001). Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus Symbiodinium using the ITS region: in search of a “species” level marker. J. Phycol. 37, 866–880.

Parkinson, J. E., Coffroth, M. A., and LaJeunesse, T. C. (2015). New species of Clade B Symbiodinium (Dinophyceae) from the greater Caribbean belong to different functional guilds: S. aenigmaticum sp. nov. , S. antillogorgium sp. nov., S. endomadracis sp. nov., and S. pseudominutum sp. J. Phycol. 51, 850–858.

Santos, S. R., Taylor, D. J., and Coffroth, M. A. (2001). Genetic comparisons of freshly isolated versus cultured symbiotic dinoflagellates: Implications for extrapolating to the intact symbiosis. J. Phycol. 37, 900–912.

Steinke, M., Brading, P., Kerrison, P., Warner, M. E., and Suggett, D. J. (2011). Concentrations of dimethylsulfoniopropionate and dimethyl sulfide are strain-specific in symbiotic dinoflagellates (Symbiodinium sp., Dinophyceae). J. Phycol. 47, 775–783.

Xiang, T., Hambleton, E. A., Denofrio, J. C., Pringle, J. R., and Grossman, A. R. (2013). Isolation of clonal axenic strains of the symbiotic dinoflagellate Symbiodinium and their growth and host specificity. J. Phycol. 49, 447–458.