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# Isolation of bacteria associated with mucus on shark skin

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## ABSTRACT

Animals and plants are metaorganisms or holobionts associated with prokaryotic and eukaryotic microbes, the diversity and community composition of which is increasingly being characterized thanks to the advent of culture-independent next-generation sequencing applications (Rohwer et al. 2002, Bang et al. 2018). In order to investigate the mechanistic underpinnings of host-microbe interactions, however, traditional culture-based approaches are critical to complement culture-independent -omics techniques. One venue for microbial cell culture-based approaches is the study of microbes associated with large aquatic vertebrates that cannot be readily studied and/or sampled under standardized and controlled conditions in the lab, such as sharks (Pogoreutz et al. 2019). The skin of sharks, rays, and skates is characterized by a mucus layer secreted by specialized secretory cells (mucous cells) in the epidermis (Meyer and Seegers 2012). Rays and skates produce sufficient amounts of epidermal mucus to be scraped off with sterile tools and collected in culture tubes, serial dilutions of which can be used for inoculation in growth media for subsequent bacterial colony picking and isolation (Tsutsui et al. 2009; Ritchie et al. 2020). Sharks however only secrete a thin and inconspicuous epidermal mucus layer in comparison (Meyer and Seegers 2012), rendering the scraping approach unfeasible. Here we detail an alternative and non-invasive protocol that allowed us to reproducibly isolate a diversity of bacteria from the skin mucus of black tip reef sharks (*Carcharhinus melanopterus*) using a swab sampling approach. Importantly, swabbing may help reduce sampling stress on sharks, as it does not require sharks being lifted onto a vessel.

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GUIDELINES

**Remarks: Direct inoculation vs. Microbial 'enrichment'**

We trialed two different inoculation approaches using sterile Difco 2216 Marine Agar/Marine Broth (according to manufacturer's instructions) and Difco Nutrient Agar/Nutrient Broth (supplemented with 3.5 % marine salts) to maximize bacterial diversity.

**(a)** Direct inoculation: directly smear the swab skin mucus sample around on the agar plate; twirl the swab while doing so to dislodge microbial cells.

**(b)** Microbial 'enrichment': add swab to an aliquot of Marine Broth or sea salt-supplemented Nutrient Broth (or other medium of your choice). Shake swab to try and dislodge bacterial cells on the swab. Depending on ambient temperatures and microbial growth rates during incubation, incubation times can be varied between 2 – 24 hrs. Depending on density of cell growth, sterile dilutions with sterile seawater (1:10 – 1:100) are recommended to prevent 'matting' growth and to ensure colony formation for picking and isolation. Diluted cell suspensions can

directly be used to inoculate marine agar plates for colony picking (e.g., 10 µl pipetted onto a 60 mm agar plate and spread out with a loop). Leave inoculated plates topside for 1 min for bacteria to settle; subsequently turn plate upside down for incubation.

**Note:** To maximize taxonomic diversity of bacteria, the direct inoculation of agar plates is preferable. The 'enrichment' approach will ultimately favor fast-growing bacteria within hours, such as *Vibrio*, which may outcompete slower growing bacteria, and may result in low diversity of colony morphologies growing on your plate.

**Note:** if specific bacterial functional groups are being targeted, use of media selecting for the specific target group is recommended.

#### **Remarks: Contamination with seawater microbes and aseptic techniques**

(1) While contamination with seawater bacteria could be a concern during sampling, results of Sanger sequencing have confirmed that the culturable diversity obtained by our swabbing approach indeed constitutes a subset of the diversity previously identified in skin 16S rRNA sequencing datasets from the same species of sharks from the same sampling location (Pogoreutz et al. 2019; Pogoreutz et al. unpublished). Notably, shark skin associated bacterial communities are distinct from those of seawater (Doane et al. 2017; Pogoreutz et al. unpublished).

(2) Although it is impossible to maintain an entire research or fishing vessel sterile, standard aseptic techniques should be employed whenever feasible. During sampling, contamination can be minimized by use of gloves that are cleaned with 70% ethanol in between steps, as well as sterile swabs. For the inoculation of media and isolation of bacteria in a lab not designed for aseptic techniques, a sterile field of work can be created by cleaning the area with bleach and 70% ethanol and the use of candles or Bunsen burners. All media, seawater for serial dilutions and tools used during this work should be sterile (i.e., autoclaved in borosilicate bottles and then carefully pre-aliquoted in a sterile environment; metal loops should be flamed between streaks). Optionally, disposable pre-sterilized inoculation loops can be used, but this will lead to the accumulation of contaminated plastic waste.

#### **Remarks: Disposal of contaminated waste and bacterial cultures**

Contaminated waste and cultures to be disposed of should be inactivated before being discarded. In the absence of an autoclave (or pressure cooker / sterilizer), the use of bleach is recommended.

#### **SAFETY WARNINGS**

#### **Remarks: Disposal of contaminated waste and bacterial cultures**

Contaminated waste and cultures to be disposed of should be inactivated before being discarded. In the absence of an autoclave (or pressure cooker / sterilizer), the use of bleach is recommended.

#### **BEFORE STARTING**

#### **Permits:**

The logistics of sampling vertebrates in the wild may differ strongly depending on species of wildlife sampled, environmental settings, available facilities, and country, and may be subject to obtaining research permits prior to sampling. In addition, sampling logistics should be designed in the least invasive manner possible, and not result in harm to wildlife.

- 1 Sampling: Collect replicate swab samples to potentially increase diversity of isolates. If sampling is vessel-based, and circumstances permit, let the skin of the shark first dry for a couple of seconds, and then swab the desired skin area (approx. 8 – 10 strokes, if feasible) using a sterile cotton swab. Use a new swab for each replicate for each location on skin. Put each swab individually back into its collection tube, and close the tube. Wrap top in parafilm if needed.
- 2 We did not use a specific transport medium, but kept the samples cool and minimized time spent between sampling and inoculation (< 1 h, sometimes rather less in our case) so as not to stress cells. If many replicate swabs are being collected and time between sampling and inoculation is a concern, use of adequate transport media at the correct salinity and pH may be considered.
- 3 Take the swab samples back to the lab as soon as possible and use to immediately inoculate medium of choice in a sterile environment (depending on facilities available: laminar flow cabinet, or open on a clean bench using

candles/Bunsen burner, if possible in a closed room and try to limit airflow) using standard aseptic techniques. Minimize the time passed between sample collection and inoculation to minimize stress for the sampled bacterial cells.

- 4 The media of choice will depend on the research question, i.e. specifically whether the aim is to maximize diversity or whether a specific bacterial functional group is targeted (i.e., a 'general' rich media vs. selective media).
- 5 Incubate plates at a slightly lower temperature than the environment they were collected/isolated from to inhibit overgrowth by fast-growing bacteria.
- 6 Incubate plates overnight, or until colony formation on the agar becomes apparent.
- 7 For the next step, do not wait until individual colonies merge, or pronounced smears/biofilms form. If colony formation is slow, keep monitoring for new growth over the course of several days.
- 8 Pick individual bacterial colonies with a clean inoculation loop (1 to 10 ul, as preferred). Streak out colony on a new agar plate using the standard Four Quadrant Streak method (Sanders 2012). Carry out at least 2 purification steps per isolate.
- 9 After purification from a single colony (min. 2 clean passages), confirm identity with Sanger Sequencing using a universal bacterial full-length 16S rRNA primer pair (e.g., 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-GGTACCTTGTACGACTT-3'; Lane 1991).
- 10 For long-term storage, consider standard cryopreservation in glycerol stocks (e.g., 25% - 40% glycerol in broth) at -80°C or -140°C (e.g., as described here <https://www.protocols.io/view/isolation-culturing-and-cryopreservation-of-endozo-t2aeqae>).