Isolation, culturing, and cryopreservation of Endozoicomonas (Gammaproteobacteria: Oceanospirillales: Endozoicomonadaceae) from reef-building corals

Claudia Pogoreutz[1], Christian Voolstra[1]

[1]King Abdullah University of Science and Technology

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Christian Voolstra

King Abdullah University of Sc...

ABSTRACT

Endozoicomonas are gram-negative bacteria widely and often abundantly associated with marine invertebrates and fish (Yang *et al.*, 2010; Bayer *et al.*, 2013; Nishijima *et al.*, 2013; Hyun *et al.*, 2014; Katharios *et al.*, 2015; Ding *et al.*, 2016; Neave *et al.*, 2017a; Schreiber *et al.*, 2016; Pogoreutz *et al.*, 2018). Despite their ubiquitous distribution, only few cultured strains are available, as *Endozoicomonas* are supposedly difficult to isolate and to maintain in pure cultures (Neave *et al.*, 2017b). Here we detail a protocol that allowed us to reproducibly isolate *Endozoicomonas* from stony corals (*Acropora humilis*) from the Red Sea. This protocol should be useful in isolating abundant *Endozoicomonas* from other corals or marine invertebrates.

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PROTOCOL STATUS

Working

We use this protocol in our group and it is working.

GUIDELINES

For the Isolation of *Endozoicomonas*, several factors are important to consider: **homogenization**, **dilution**, **incubation temperature**, **replication**, and **colony morphology**:

(1) **Homogenization**: Thoroughly homogenize the coral tissue to make sure the Endozoicomonas aggregates are 'set free' from the host tissue (e.g., for 30 seconds at medium speed with an UltraTurrax or comparable tool). We didn't do that in a first attempt and all we isolated was Vibrios. Once we implemented the tissue homogenization, overall taxonomic diversity of isolates increased.

(2) **Dilution**: if the Endozoicomonas make up a high proportion of the microbiome (for instance, 60 - 90 % in Red Sea Acropora humilis), the dilution will help to select for abundant organisms. We have plated tissue slurry "undiluted" and in 1:10, 1:100, 1:1000 dilutions. The Endozoicomonas grew on the Marine Agar plates inoculated with 'undiluted' and 1:10 diluted slurries.

(3) **Temperature**: we settled for an incubation temperature low compared to ambient seawater temperatures (23°C in our case; the central Red Sea has an annual temperature average of 29°C). At this temperature, Vibrios, Alteromonas, and other fast-growing taxa would form colonies within the first 24 h, while the Endozociomonas started to appear on day 4 post-inoculation, but would form many more colonies than other taxa.

(4) **Replication**: we had an agar plate replication of n = 3 for each dilution of slurry and type of media (other media than Marine Agar were used, too, but no Endozociomonas grew on them). This was not entirely necessary in our case because we had hundreds of Endozoicomonas colonies growing, but it might increase the chance of isolating Endozoicomonas strains (or other bacteria) that are not quite as abundant.

(5) **Colony morphology**: Endozoicomonas will form tiny round convex creamy colonies with entire margins. When plating the picked colonies, you will see that the average Endozoicomonas colony is rather sticky and will strongly adhere to loops or needles. These bacteria are certainly easier to handle in suspension culture than on plates. Once you have successfully isolated Endozoicomonas on plates and confirmed their identity with Sanger sequencing, you may transfer them in liquid culture (Difco2216 Marine Broth).

1 Collect coral fragment(s) (approx. finger-sized) in a sterile zip-lock bag. Take notes on sampling conditions (site, sampling depth, habitat).

2 It is recommended to process coral fragment(s) right away. If not feasible, maintain at ambient reef water temperatures in flow-through aquaria (Temperature 28°C, salinity 40 PSU) or in closed aquarium systems with daily water exchange.

3 To obtain coral tissue slurry for inoculation, blast tissue off the coral skeleton using an air gun and autoclaved filtered seawater (AFSW; Whatman, 0.22 um).

4 Homogenize a total volume of tissue slurry of no more than 15 ml per finger-sized fragment for 30 sec (e.g., using an IKA UltraTurrax).

5 Plate 50 ul of slurry on Marine Agar 2216 (MA; BD Difco) undiluted and in 1:10 dilution in triplicates. After incubation at 23°C for 4 days, Endozoicomonas colonies will form and should be purified starting from a single colony.

6 After purification (minimum of 2 clean passages), confirm identity with Sanger sequencing.

7 Inoculate 8 ml Marine Broth 2216 (MB; BD Difco) with a 10 ul loop of cells from purified colonies on MA. Close the tube, vortex, and incubate and under constant motion (e.g., 60 rpm) at 25 ˚C for 48 h.

8 Snap-freeze aliquots of Endozoicomonas strain in suspended culture as a 20% (v/v) glycerol suspension in MB and store at -80 ˚C or -140 ˚C.

9 Note: for the isolation of low abundance Endozoicomonas or other bacteria, (1) the amount of AFSW used should be kept to a minimum or no AFSW should be used at all to not unnecessarily dilute the slurry, (2) a selective medium might be required.