

TITLE: Microbial Symbionts from the Endostyle Region of the *Ascidella aspersa* (Sea Squirt)
capable of metabolizing iodine?

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Abstract: Tunicates (sea squirt) are filter feeders which process liters of sea water daily. They have a preemptive organelle similar to the vertebrate thyroid called an endostyle. The endostyle has been identified as site for iodine metabolism. Our hypothesis was that microbial symbionts associated with endostyle play a role in iodine metabolism. Using *Ascidella aspersa* (sea squirt) as the model, we have identified *Endozoicomonas ascidiicola* as a dominant symbiont of the endostyle and its surrounding tissues. We have found that the mucus secreted by the endostyle in the bronchial sac has an acidic pH providing a unique microbial environment. Indirect evidence from fluorescent microscopy suggest the *Endozoicomons* are present in tissue and not in the mucus layer and warrant further investigation.

INTRODUCTION:

The mechanism of uptake of iodine have been characterized in the thyroid gland of vertebrates, brown algae, and kelp. However, the microbial contribution of this uptake has never been studied in tunicates. This led to our hypothesis “Does the sea squirt, a tunicate, harbor microbial symbionts capable of metabolizing iodine/iodate from the sea water?” The sea squirt can filter hundreds of liter of sea water per day where iodine is primarily present in the form of iodide (I-) or iodate (IO₃-). The concentration of iodide is higher in warm surface water (0.10 pg per liter on the average) than that in cold deeper water of lower temperature than 20°C (0.03pg per liter) (Tsunogai and Henmi, 1971). The Zone 5 (immediately above the dorsal glandular tracts) of the Endostyle of tunicates contains organically bound iodine in the form of monoiodotyrosine, diiodotyrosine, and thyroxin. It has been shown that endostyle extracts from *Ciona intestinalis*, contains organoiodo compounds (Barrington, 1958; Barrington and Thorpe, 1965).

To address our hypothesis, the first step was to establish the presence of symbiotic organisms that are in close association with the endostyle of sea squirt. Secondly, to screen for organisms capable of oxidizing iodide in culture.

MATERIALS AND METHODS:

Sampling: The sea squirt samples were collected from Sandwich Marina, near Cape Cod Canal, MA, (GPS coordinates: 41.770970, -70.503838). During sampling the pH, salinity and temperature were measured using Professional plus YSI series. Samples were collected as needed and maintained in a sea

water table with running filtered sea water.

Dissection: The sea squirts were carefully dissected and the endostyle was exposed (Figure 1). The endostyle along with 2mm of surrounding tissue was isolated.

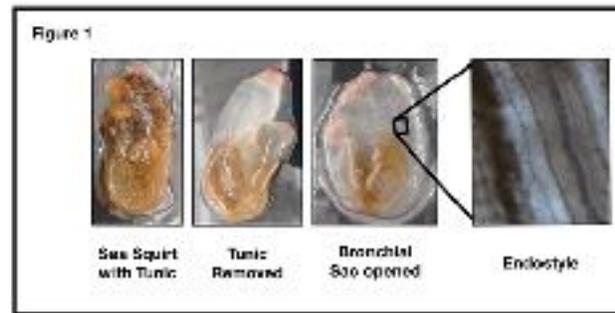


Figure 1. Dissection of *Ascidiella aspersa* for Endostyle

Culture: For differential counts, artificial sea water complete medium was prepared. For this, 1X Sea Water Base (NaCl, 342.2mM; MgCl₂.6H₂O, 14.8mM; CaCl₂.2H₂O, 1mM; KCl, 6.71mM) was mixed with 5g Bacto trypton, 1g yeast extract, 3ml glycerol, 5ml MOPS (pH 7.2) and 15g agar per liter, and autoclaved. For enrichment under acidic condition, three different kind of liquid media were designed using 1X Sea Water Base 1) N-acetyl Glucosamine 2) Pectin, and 3) Complete The pH was adjusted to 2.5 using 10N H₂SO₄. All medium were filtered sterilized using 0.22 μ filter and aseptically dispensed in the sterile culture tubes.

The endostyle tissue was rinsed three times with sterile Sea Water Complete (SWC) and homogenized in 1ml of SWC. The serially diluted endostyle homogenate was used as inoculum.

The pH determination in the bronchial sac/ acid mucus of sea squirt: The internal matrix from the endostyle and immediately surrounding area was scrapped and resuspended in pure water (pH 8.0), the pH was measured using a pH electrode.

Microscopy: The endostyle after dissection was immediately processed for **Scanning Electron**

Microscopy (SEM): The samples were fixed for 2 hours in 4% formaldehyde in 1X phosphate buffered saline (PBS, pH 7.2). Washed three times with 1XPBS followed by serial dehydration with increasing concentrations of ethanol (25%, 50%, 75% and 100% ethanol) prepared in pure water (PW). The samples were dehydrated twice for 10 minutes each, with increasing concentration of ethanol and transferred to the critical point drying machine - Samdri-780 A. After drying the samples were carefully placed on stubs and sputtered coated with 10nm layer of platinum in vacuum and observed under SEM.

For Fluorescent Microscopy: The live/dead bacterial stain was prepared by mixing Syto9 and propidium iodide in 1:1 ratio as per manufacturer's instructions (Thermofisher Inc.). A small piece of tissue was placed on a glass slide along with premixed stain (in 20ul volume), a coverslip flattened the soft tissue sample. The cover glass was sealed with nail polish and observed at 100X (Zeiss Axio 2.1).

Genotyping: Briefly, a small piece of tissue was collected for three sea squirts (A-C), DNA was extracted using ZYMO genomic DNA isolation kit as per the manufacturer's protocol. The extracted DNA was

quantified using Quantus fluorometer (Promega Inc.). The PCR was set up as described by Pe' rez-Portela et al., 2013. The primer set used are for the gene Cytochrome Oxidase Subunit I (Tun_forward, 5' TCGACTAATCATAAAGATATTAG 3', and Tun_reverse2, 5' AACTTGTATTTAAATTACGATC 3). The resulting PCR was sequenced using Sanger sequencing method. **Colony PCR for microbial isolate identification:** Colony PCR was performed to ID the select few colonies from the enriched culture. Briefly, bacterial colonies were picked and resuspended in 20µl Pure Water and boiled for 10 minutes at 95°C, 2µl of supernatant was used for PCR using 8F- AGAGTTTGATCCTGGCTCAG and V8R GACGGGCGGTGWGTRC primers. The resulting PCR product was sequenced using Sanger sequencing. The sequences were blasted using www.ncbi.nlm.nih/BLAST tool for organisms' identity.

RESULTS

Differential Count: For this, 2-1 cm long endostyle was homogenized in 1ml of SWC (pH 7.2), serially diluted and 100µl was plated on SWC medium (pH 7.2). After 72 hour of incubation small pin head shaped colonies were observed (Figure 2). As shown in Table 1, We observed $\sim 1.23\text{-}1.38 \times 10^5$ CFU/cm³

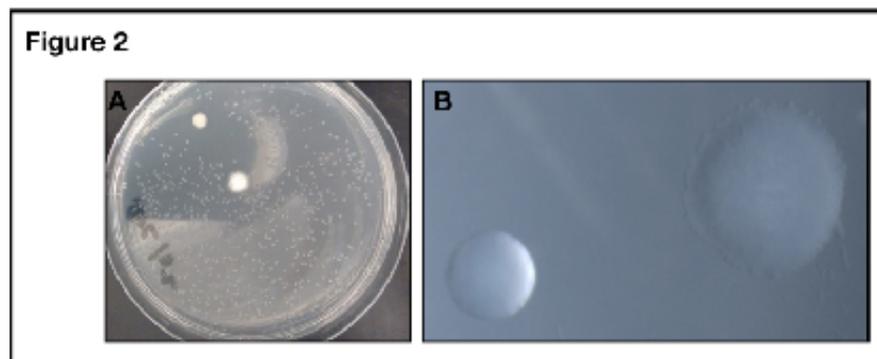


Figure 2: Colony morphotype for enrichment culture from Sea Squirt Endostyle

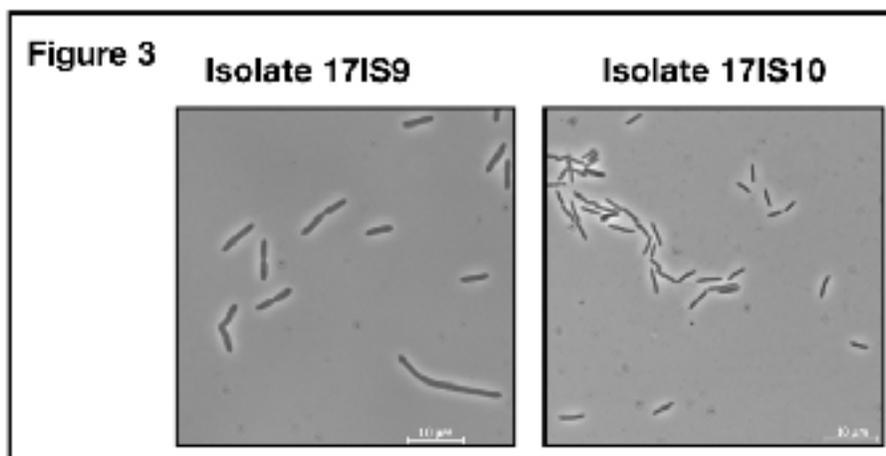


Figure 3: Phase contrast microscopy of select isolates from endostyle homogenate culture identified as *Endozoicomonas sp.*

from three individual sea squirts. This suggests that all sea squirts had endogenous bacteria in the tissue in the similar amount. The wet mount phase contract microscopy (100X, oil immersion) of select colony isolates shows rod shaped morphology (Figure 3).

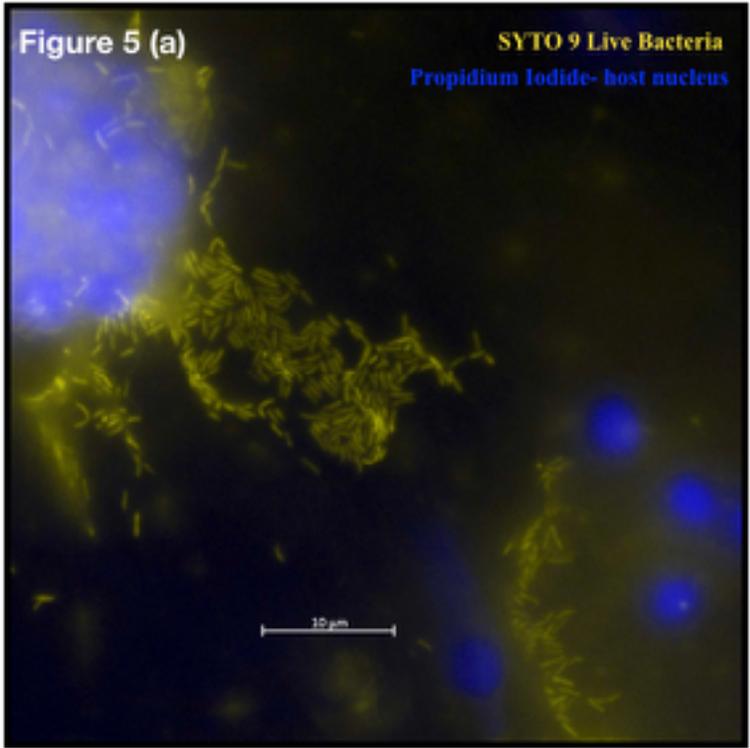
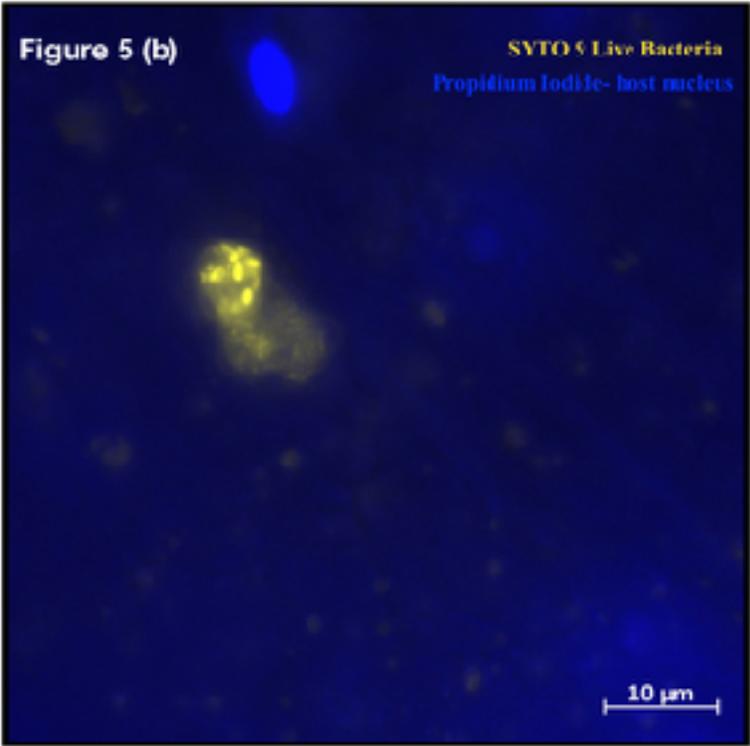
Measurement of pH in Endostyle Matrix: For this 1 ml of scraped matrix was suspended in 5ml of pure water. The resulting mixture was vortex and the pH was measured using calibrated pH meter. The pH of this mixture was 2.52, suggesting a highly acidic environment.



Figure 4: Scanning electron micrograph showing endostyle (ES) from three different squirt (ESA, ESB, and ESC).

Microscopy: Endostyle from four individuals were rinsed in a sterile SWB (pH 7.2) to remove the loosely attached microorganism followed by immediate fixation. The data shown in figure 4 is inconclusive as we did not observe bacterial colonization on the surface. For fluorescence microscopy, the endostyle tissue was subjected to live/dead bacterial staining using SYTO9/ propidium iodide (PI). SYTO 9 and PI stains bacteria with intact cell membranes stain fluorescent yellow, whereas bacteria with

damaged membranes stain fluorescent red. As shown in Figure 5 (a-c) live bacterial cells were observed within a small section of endostyle of a single sea squirt.



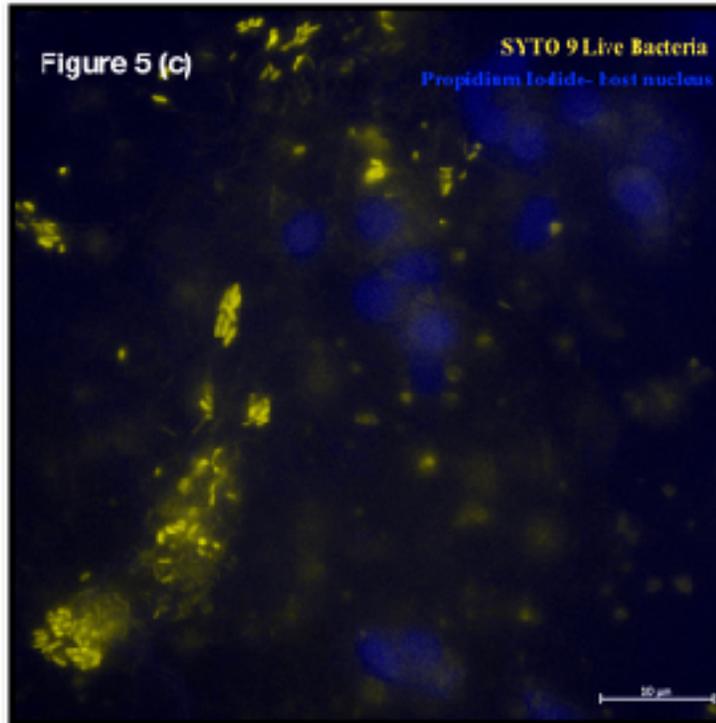


Figure 5: Fluorescent microscopy of endostyle tissue for the presence of endosymbionts in the tissues. A -C live and dead bacterial staining using SYTO 9 and propidium iodide. Yellow staining depicts live bacteria while blue indicate staining of host nuclear cells

Genotyping: The 16S sequencing analysis of three isolates from homogenized tissue showed 99% identity to *Endozoicomonas ascidiicola* (Table 2). The genotyping of sea squirt using tunicate specific COI primers identified the model organism as *Ascidiella aspersa*.

Table 2: Sequence identify of isolates from the endostyle of sea squirt

	Source	Identity	Identiy
17IS09	SQC	100% (602/604)	Uncultured bacterium clone Woods-Hole_a5893 16S ribosomal RNA gene
17IS10	SQD	100% (590/591)	Endozoicomonas ascidiicola
17IS11	SQB	100% (602/604)	Uncultured bacterium clone Woods-Hole_a5893 16S ribosomal RNA gene

DISCUSSION AND CONCLUSION:

The phylogenetic analysis of sea squirt using tunicate specific COI primers identified sea squirt as *Ascidiella aspersa*, The *Ascidiella aspersa* (European sea squirt) is a solitary marine and estuarine

tunicate that is native from Norway to the Mediterranean. It is an invasive species for northeastern Atlantic region. Rather than characterizing the microbial community structure associated with the endostyle, we focused on the enrichment of symbiotic microorganism present in the endostyle of sea squirt. The homogenized endostyle tissue was serially diluted and plated on SWC medium. We identified in all of the four sea squirts that are part of this study, the most common colony morphotype as *Endozoicomonas ascidiicola*. *Endozoicomonas* sp. is a gammaproteobacteria which belongs the family Hahellieacea. Most of the bacteria in this group are either pathogens or ecologically important. In 2016 Schreiber et al, showed that *Endozoicomonas* is a facultative symbiont of ascidians. They also showed that it is horizontally transferred.

We found that pH in bronchial sac of sea squirt is highly acidic (pH <2.52). The preliminary fluorescent microscopy and mucus pH data suggested *Endozoicomonas* sp. primarily resides in the tissue of sea squirt and not in the acidic environment of the mucus. This is in conflict with the findings presented by Schreiber et al., in 2016 where they suggested that *Endozoicomonas* live off the mucus continuously secreted by the pharynx **without** affecting the ascidians host. They also proposed that mucus degradation and metabolism also plays a role in *Endozoicomonas*-host system. Similarly, other groups have identified the microbial community structures associated with tunic, pharynx and gut of the sea squirt, *Ciona intestinalis* (Dishaw et al., 2014; Blasiak et al., 2014). There was no discussion of an acidic environment. We suggest that such interaction should be carefully investigated in relation to pH of the mucus secreted by the host cells. We believe that the acidic mucus is characteristic of ascidians and other gelatinous marine organisms that are filter feeders. In another ongoing study of doliolids, (also filter feeders in the open ocean) the attempt to study microbial community structure in the gut failed (personal communication). This could be because of acidic pH in the bronchial sac that was never looked at. The acidic environment is potentially protective, prevents biofilm formation, helping to trap food particles in the mucus, and partially hydrolyzing the food under acidic conditions. However, we cannot rule out that there might be acidophilic microorganisms residing in such an environment. The scanning electron microscopy data was inconclusive as we did not observe any bacteria on the surface of endostyle from three different sea squirt individuals.

Future Direction: This study leads to many questions that need to be answered 1) Is *Endozoicomonas ascidiicola* located intracellularly or intercellular as aggregates? This can be done by using either transmission electron microscopy or confocal microscopy; 2) Are there other organisms present? 3) Is *Endozoicomonas* sp. acid tolerant or sensitive?; and 4) Is the bacteria capable of iodide oxidation and/or iodate reduction under aerobic and/or anaerobic conditions?

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