

## Tunicates and Bacteria: Food, Commensals, Symbiosis...

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### Introduction:

Ascidians (tunicates or 'sea squirts') are sessile, filter feeding marine urochordates. Symbiotic relationships between ascidians and prochloron and synechococcus have been documented, and a number of other observations suggest more possible symbiotic relationships exist. Additionally a number of species of tunicates are known to concentrate vanadium in a reduced form to  $10^7$  times the concentration found in seawater (where it is oxidized). The purpose of this concentrated reduced vanadium is unknown. A number of tunicate species occur in Eel Pond at Woods Hole, growing in large numbers on dock floats. At least six species grow on top of each other on these floats, covering 60-80% of the float surface. This study addresses three questions: 1) do these co-occurring species have different food sources, 2) are particular non-food bacteria associated with each tunicate species, and 3) are the bacteria from high vanadium tunicates more resistant to high vanadium levels than those from the low vanadium species.

Tunicates are 'protochordates', exhibiting chordate-like morphology only in the larval stage. Some species of tunicates have motile, tadpole-like larvae with a stiff notochord within the tail. Larvae attach to a substrate and metamorphose into a sessile adult that looks more like a sponge or mollusk than a chordate. Adult tunicates are filter feeders, bringing water in through an incurrent siphon and straining it through a pharyngeal basket. Water and waste leave the body through an excurrent siphon. Tunicates can be solitary, social, or colonial. Colonial tunicates share the outer leathery surface (the test) as well as excurrent siphons. Social tunicates have distinct zooid bodies with individual tests and siphons, but are connected by a stolon that carries food and nutrients. Solitary tunicates are generally the largest type. (See first picture)

Tunicates (*Didemnum* and other species) have in the past been shown to have obligate bacterial symbionts (prochloron and synechococcus) in their cloacal cavities or embedded in the test matrix. Additional evidence for bacterial associations or symbioses in these animals is microscopic observations of associated bacteria, and the isolation of bacterial or fungi-produced chemicals of pharmaceutical use from tunicate samples.

Vanadium concentration is seen in the local tunicate species *Perophora*, which is a high-level vanadium concentrator, as well as at more moderate levels in *Ciona intestinalis*. Vanadium concentrations in other local species are either low or unknown. *Perophora* concentrates V (iii) while *Ciona* concentrates V(iv). Vanadium, which has five oxidation states, is found in seawater at low concentrations (.0025 ppm) and in the oxidized state V(v). Vanadium within the tunicate is found within vanadocytes (which may also contain sulfuric acid) in the blood, though it is also sometimes found on the surface of the test. Very little is known about vanadium in biological systems, though it is found in some nitrogenase enzymes as well as a few bacterial haloperoxidases. The purpose of the high vanadium concentrations in tunicates is unknown, but it may serve as a deterrent for predators or for organisms that would grow as epibionts on the tunicate test. Vanadium has been shown to be toxic in laboratory rats at a concentration of 200ppm, but little is known of its toxicity in bacteria.

## MATERIALS AND METHODS

### Collection site:

A number of different tunicate species are easily found in the Woods Hole area, often growing on dock pilings, sea grass, and also on sandy seafloor. After surveying occurrence of tunicates on a number of dock pilings in the area, I found that the best collection sites were the Styrofoam floats on Eel Pond floating docks, rather than the wood pilings. All local collections were taken from such floats underneath the floating wood dock closest to Swope building. At least six tunicate species were present on the sides and undersides of these floats, growing in close proximity or even on top of each other, and representing most (~60-90%) of the biomass and biotic coverage of the float surface. Both solitary and colonial tunicates were present. Also present were a number of different bryozoan species, at least one sponge species (in low number), and motile fauna such as amphipods, nematodes, daphnia, etc.

### The animals:

The following tunicate types were identified:

*Styella* sp – A large (8-10cm) stalked solitary form with a very thick (5+mm) and tough test. Siphons have six reddish stripes, and test is warty and wrinkled, ranging from pale yellowish in the thinner wrinkled sections to a reddish brown in the thick warty sections. Many epibionts present on test: bryozoans (bushy and encrusting), botryllus and the orange colonial tunicate, as well as small *Ciona intestinalis*. The white firm colonial was sometimes found associated with the stalk attachment. Body is firmly encased in the tough test, and is difficult to dissect. When dissected from the test, the entire body is a bright orange color, and inhabits roughly  $\frac{1}{3}$  to  $\frac{2}{3}$  of the entire test length.

*Ciona intestinalis* – also a large (6-8cm) solitary tunicate. A transparent to yellowish test, through which the pharyngeal basket, intestines, and gonads can be seen. Test attaches to substrate with fleshy finger-like protrusions. Very little to no epibiotic growth seen on this species, although young members of same species, and also of unknown solitary were seen growing on bottom portion. Siphons have a bright yellow rim. Test is thin (<1mm), smooth, and easily cut or ripped, and body is only loosely associated. The body inhabits most of the test. Digestive tract is mostly clear, though brown green when full of waste.

Unknown solitary – (another invasive??) an unstalked solitary reaching roughly 3cm. Siphons are very short (excurrent seems flush with test wall, and is found roughly  $\frac{1}{3}$  of body length from toward base), and have a red speckled pigmentation that varies between animals. Red pigmentation outlines 8 points around siphon. Test is tough but thin and easily cut, warty and semitransparent. Body inhabits most of the test. Intestinal tract is thick and large for body size (1.5 cm long, 3-4mm thick), and outer wall of digestive tract is thick and brown pigmented; the interior is bright orange. A fair number of epibionts present, including most often nonbranching bryozoans and the white fleshy colonial tunicate, which in one of the four samples had grown completely over the two siphons.

The orange invasive tunicate common in area (“Freeman’s Blight”) – a bright orange colonial with many pigments in tunic. Tends to grow on any available surface, but most often seen growing directly on the dock float or on *Styella* tunics.

Botryllus sp – star shaped colonies with variable pigmentation (light on dark, or dark on light). Tunic matrix is fairly soft. Tends to grow on *Styella*, and also on available dock float surfaces.

Unidentified colonial (at first mis-identified as *Didemnum* – *Aplidium*?) - Thick gel like whitish matrix with embedded zooid bodies. Colony could be 1cm thick from top to bottom, and several (~3cm) in diameter. Zooids were roughly 2mm long, and had bright orange pharyngeal baskets, greenish intestine, and pinkish glands (probably gonads). Colonies grew on dock floats, on the base of *Styella* stalks, and on the surface of the unknown solitary tunicate.

*Perophora viridis* – this species was obtained from the Marine Resources Center, and is not from Eel Pond. It is a social tunicate that grows on various algae. Zooids are independent, roughly 1mm in diameter, and occur at regular intervals along a basal strand that runs the length of the attachment substrate. Tunic is thin, lightly brownish/transparent. Pharyngeal basket is orange, and some zooids, as well as the shared basal strand, were green-tinted.

#### Food Sources:

#### Microscopic analysis:

To study the food sources used by the different tunicate types (colonial and solitary) and species, waste was collected from the three larger solitary species and observed under the microscope. In the colonial species fresh samples were dissected and either whole bodies or digestive tracts were placed on slides for observation. Extra water for wetting slides was unfiltered seawater from the specimen dish.

#### Culturing and t-RFLP:

##### Basic medium:

##### Jared's artificial seawater

DI water	+NH <sub>4</sub> Cl
Yeast extract	NaSO <sub>4</sub>
Peptone	trace metals
(agar if for plates)	12 vitamins

Waste from the larger animals was collected by placing a single freshly collected individual (one of each 3 solitary species found) in a specimen dish with clean (not filtered) sea water. Animals excrete waste as coherent packages that are ejected from the excurrent siphon. Waste packets were collected with tweezers within 10 minutes of ejection. Roughly 100uL volume of waste from each animal was placed in a microfuge tube, and 1 mL of 0.2um filtered seawater added. These tubes were then vortexed to break up and resuspend waste, and one drop of each was either plated (SWC, low V, med V, high V) or added to liquid media (SWC, med V, high V). The remaining volume was frozen for later t-RFLP analysis.

DNA was extracted from the frozen resuspended waste using a MoBio UltraClean Soil DNA Isolation Kit, which involves bead beating to break open cells, and a series of washes to remove contaminants. DNA was eluted from the extraction filter with 50ul of the provided elution buffer (no EDTA). This DNA was then used in a t-RFLP PCR using a bacterial specific primer (SDBact0008F20) which carried a fluorescent tag, and a universal untagged 16s primer, 1391R, with the following amplification reaction and parameters:

##### Reaction mix:

17ul	dH <sub>2</sub> O
2.5 ul	10x buffer
2 ul	25 mM MgCl <sub>2</sub>
1ul	2.5 mM each dNTPs
1ul	8F bacterial primer (with fluorescent tag)
1ul	1391 universal primer
0.25ul	Taq polymerase

##### Thermocycler parameters:

95 degrees for 5 minutes, then the following with 25 cycles: 95 melting for 30 seconds; 55 degree annealing for 30 seconds; 72 degree extension for 1 minute.

Medium vanadate plates (from the first autoclaved batch) grew a mix of green and white colonies, the proportion of which varied between species. Media in these plates also began to turn dusky green over time. *Styella* and *Ciona* homogenates both turned the plates green, and also had the highest proportion of green colonies. The unknown had a large number of colonies, most of which were white, and did not change the plate color. *Perophora* homogenate, which had fewer colonies in general, had a few large green colonies, but failed to turn the plate green. *Styella* and unknown waste cultures grew many colonies on Medium plates, but had very few green colonies. *Ciona* waste only grew eight colonies on Medium culture plates, and six of the eight were green. None of these plates had an overall color change.

t-RFLP: No results back at the time of this paper...

## **DISCUSSION:**

### **Food:**

From microscopic observations the bulk of tunicate food consists of diatoms, algae, and filamentous cyanobacteria. Live motile bacteria were found in food particles which may either be undigested bacteria or part of the gut microbial population. Similar motile bacteria were seen in each species, though whether or not they were identical could not be said. Cultures of *Styella*, *Ciona*, and the unknown waste on seawater complete plates grew many colonies from each animal, as did liquid cultures. Plates contained 2-3 different colony morphologies, and appeared similar to each other. Liquid cultures also appeared similar to each other, containing motile ovoids and spirilla. The t-RFLP results were not back at the time of this report, and therefore little can be said about whether these cohabiting tunicate species share a particular bacterial food source, though from microscopic and culture work it appears that both food source and some intestinal flora may be similar or identical between all species.

### **Nonfood associated bacteria:**

No bacteria were seen associated with the tests of those animals studied, nor with the pharyngeal basket, though this often contained many particles which could have been bacterial, but were more likely pigment particles or some other eukaryotic cell fragment. Bacteria were seen in the digestive tract and within a sample which may have been a blood vessel. Bacterial populations changed with position with the intestine, as well as with presence or absence of food. The cloacal region where prochloron has been seen before was not investigated due to difficulty and inexperience in tunicate dissections (I couldn't be sure exactly what I was looking at for much of the animal).

Plates from homogenized tissues on seawater complete medium showed very high growth, with many fast growing colonies. Colony morphologies were not the same between plates, with some plates containing spreading colonies, some yellow round colonies, etc. Liquid seawater complete cultures also had high growth, and contained populations of bacteria that appeared different in the different inocula. Again, t-RFLP analysis was not complete at the time of this report, but from culture and plates it appears that different bacteria are associated with the different species. Of course in both this and the waste cultures above, only one individual of each species was used, and only one plate or tube of each culture medium generated. The variation seen could be an animal to animal variation, not a species to species variation.

### **Vanadium:**

Though *Perophora* and *Ciona* are the only species known to concentrate vanadium, cultures from tissue homogenates of all species, as well as from waste and from Eel Pond water, grew in all vanadium concentrations tested, though waste cultures grew more slowly, and no growth was seen in the high cultures in the first 2-3 days. Medium and High vanadium cultures initially showed reduced motility (cells that were motile moved slowly) and cells often were deformed, and appeared herniated or otherwise misshapen. Later observation of the same cultures often showed an increase in motility and in proper structure of cells.

The High level of vanadium appeared to interfere much more than the Medium amount (which was \_ the High concentration) in that cell growth and motility was delayed longer in these cultures, and that the number of types of cells seen in each population of cells was much reduced from that in SWC or even Medium media.

All batch culture tubes observed changed to a dark dusky green within three days of inoculation, regardless of inoculum source. Inoculum source did however affect the speed at which the color change was seen. This green color did not appear in uninoculated media, and could not be replicated by acidification of 5ml of 1%  $\text{NaVO}_3$  (in distilled water) with one drop of 12N hydrochloric acid (this treatment turned the media bright yellow) One drop of ascorbic acid added to the same volume of 1%  $\text{NaVO}_3$ , however, turned the solution dusky green. As ascorbic acid is a reductant, the conclusion is that something within the above culture tubes is reducing the  $\text{VO}_3$ . Green culture medium was spun down to determine if the green color was associate with the cells. A low speed centrifugation generated a white cell pellet and a clearer green supernatant. Higher speed centrifugations failed to change the color of the supernatant. Plate culture of these same inocula on  $\text{VO}_3$  containing plates showed both white and green colonies, indicating that the production of green color is not the result of general metabolic processes, but is rather related to particular bacteria within the culture. Transfer of these colonies to new plates failed, and examination of the cell make-up revealed a mixed population of cells that were predominantly vacuolated, sick looking thick bars or rods. Older colonies began to lose their color after 4 or 5 days. The autoclaved media that turned green also slowly lost its color over the course of 3 to 4 days. Most likely the loss of color in these colonies is the result of auto oxidation of the reduced vanadium generated by living cells after the cells begin to die off.

The secondary loss of green color seen in the Medium tubes is more difficult to explain. These tubes maintain a green layer 4-5mm thick at the air interface, and are yellowish underneath, with a brown precipitate at the bottom of the tube. Cell die-off and auto oxidation of the vanadium would not generate or maintain a green layer at the air interface of the tube, and observation of the cultures within this tube show many living cells. The brown precipitate at the base is not crystalline, nor does it look like clumps of dead cells. It precipitates from a vortexed culture at very low speeds, and before the formation of a cell pellet. It is possible that organisms exist within this zone that can oxidize the reduced vanadium formed initially in these cultures. Here is the only evidence for a possible vanadium-related tunicate:bacterial association. All vanadium tubes turned green, but those from *Perophora* and the unknown tunicate homogenates turned green the slowest. Yet this secondary color change was fastest in those same species, and *Perophora* (a Viii concentrator) is the only species that generated a secondary color change within ten days. *Ciona*, which also concentrates vanadium (Viv) in moderate levels, showed similar color change speed (and number of green colonies) to *Styella*, which does not concentrate vanadium. It would be interesting to learn whether this unknown tunicate also concentrates V(iii).

While vanadium in general did not inhibit cell growth at medium and low levels, it does indicate that the bacterial populations within the tunicates are not identical between species, as seen by the speed of color change in batch culture, and of the proportion of green colonies on plates. Homogenates and waste also differed from each other, even from the same animal. Waste tubes generally showed a much slower color change, and fewer green colonies than the animal homogenate plates, which probably reflects differences in bacterial populations in these different sources.

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