Classification And Initial Characterization Of Symbiotic Chlorella Sp. Isolated From The Freshwater Sponge, Ephydatia Muelleri

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CLASSIFICATION AND INITIAL CHARACTERIZATION
OF SYMBIOTIC CHLORELLA SP. ISOLATED FROM
THE FRESHWATER SPONGE, EPHYDATIA MUELLERI

Submitted in Partial Fulfillment of the
Requirements for Graduation with Honors
in the Department of Biology and Chemistry,
Carroll College, Helena, Montana

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March 30, 1994
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March 30, 1994
Table of Contents

Abstract..................................................... ii
List of Illustrations......................................... iii
Introduction.................................................. 1
Literature Review............................................. 2
Materials and Methods....................................... 10
Results........................................................ 16
Discussion and Conclusions................................. 23
Works Cited................................................... 26
Abstract

A population of archeocytes was isolated from the freshwater sponge, *Ephydatia muelleri*, using a Ficoll gradient. From these archeocytes an endozoic *Chlorella* sp. was removed and cultured. The cultured *Chlorella* sp. were then used to investigate the uptake of symbiotes by *E. muelleri*. The cultured algae were added to germinated gemmules. The outgrowth from the gemmules was then examined for algae uptake by the sponge cells using brightfield and phase contrast photomicroscopy. The results were inconclusive because algal symbiotes inhabited sponge cells of both the experimental group (the sponges exposed to the algae culture) and the control group (the sponges not exposed to algae culture).
List of Illustrations

Fig. 1a  Archeocyte containing multiple vacuoles........................................18

Fig. 1b  Wide shot of Fig. 1a showing another archeocyte............................18

Fig. 2  Chlorella sp. cells isolated from archeocytes...............................19

Fig. 3  Other forms of algae in culture...............................................19

Fig. 4  Gemmule after germinating.....................................................20

Fig. 5  Archeocytes photographed at 1000x phase contrast..........................20

Fig. 6  Archeocyte at 1000x phase contrast from algae cultured gemmules.........21

Fig. 7  Photograph of the periphery of a gemmule with two archeocytes free of the cell mass..........................................................21

Fig. 8  Growth chart of Archeocyte derived algal culture..........................22
Introduction

In the clear lakes of the Montana Rockies there exists a curious group of creatures that the average angler might overlook. Yet, the inconspicuous nature of these animals gives no indication of their character. Within these plant-like animals resting on the rocky bottom of the Salmon Lake there is a complex and fascinating relationship between a primitive invertebrate, the freshwater sponge of the Phylum Porifera, and a primitive autotroph, the unicellular green algae of the genus Chlorella. These two organisms live in a fascinating harmony which has been termed "symbiosis".

In my investigation of this relationship I had two objectives. They were (1) to isolate, culture, and characterize the symbiotes, Chlorella sp., of the freshwater sponge, Ephydatia muelleri, and (2) to investigate the uptake of Chlorella sp. (its cultured symbiote) through photomicrographic analysis.
Freshwater Sponges

The members of the phylum Porifera, commonly termed "sponges", occupy a unique and surprisingly broad niche in aquatic ecosystems. Sponges, often thought of only as a marine life form inhabiting the ocean floors of coastal waters world-wide, are among the most primitive animals on the planet. Porifera express a unique form of organization in that their highest mode of specialization is at the tissue level (Frost, 1991), meaning simply that the sponges lack all organs. Not only are the organs absent, but sponges lack the three primary tissue layers (ectoderm, endoderm and mesoderm) indicative of most animals (Villee, Walker, and Barnes, 1984). In their place, sponges have an external epidermal layer surrounding a gelatinous, inner core called the mesohyl (Frost, 1991). All the necessary biological functions such as feeding, reproduction, defense, and support are carried out through a intercellular system based on specialized cells functioning together. This intriguing system has caused some researchers to consider Porifera as a colony of cells rather than a true organism (Frost, 1991).

The cell layers of the sponge are made up of several basic specialized cells: the archeocytes, the choanocytes, the sclerocytes, and the pinacocytes. The outer covering
of the sponge, the pinacoderm, is made up of a sheet of cells called pinacocytes (Frost, 1991). This cell layer is perforated by numerous orifices, called ostia, of about 50um in diameter (Frost, 1991). Ostia act as an incurrent sieve which allow water to enter the sponge's filter-feeding system, but prevent the passage of large particles that the sponge cannot utilize (Frost, 1991).

The interior cavity of the sponge is the location of the filter-feeding mechanism. Frost (1991) describes the filter-feeding mechanism as follows: a large subdermal cavity beneath the ostia leads to incurrent canals, the incurrent canals then divide into multiple constricted cavities called choanocyte chambers, the choanocytes lining the chambers create a water current throughout the water-processing system by the beating of their flagella, after the water passes through the choanocyte chambers, the many channels of water then flow into one-another, and out the large excurrent siphon.

Choanocytes consume large amounts of the filtrate, brought in by the flagellar current. This is accomplished by producing a smaller vortex within their collar of microvilli. The particulates caught by the collar of the choanocyte are then enveloped by endocytosis. Once contained within vacuoles, the ingested material may then be digested for use in the cell or passed off to the archeocyte located in the mesohyl.
The archeocyte is an ameboid cell with the capacity to form all other types of sponge cells (Villee et al., 1984). The archeocyte also acts as a primitive circulatory system in which nutrients may be passed on to other cell populations by traveling through the mesohyl. In addition, the archeocyte acts to further digest the material within the vacuoles it contains.

The last major cell type of the adult freshwater sponge is the sclerocyte. The sclerocyte is responsible for the formation of the sponge's silica skeleton, its most conspicuous and well-known feature. The skeleton is formed by the adhesion of spicules that are bonded by a form of collagen called spongin, particular to Porifera (Frost, 1991). Three different spicules are formed by these cells. The most common form of spicule is the megasclere, which is the major structural component of the skeletal system (Frost, 1991). The next form is the microsclere, which is used to further support the sponge (Frost, 1991). The last form is the gemmosclere which is not found in the adult, rather, it is a structural component of an asexual reproductive structure called the gemmule. The morphology of the gemmosclere is a primary indicator of sponge taxonomy (Frost, 1991).

The reproduction of the sponge has both a sexual (Van de Vyver and Willenz, 1991) and an asexual component. In freshwater sponges, asexual reproduction accounts for (4)
most of a sponges offspring (Van de Vyver and Willenz, 1991). Sponges may reproduce asexually by simply breaking apart into separate units or by forming specialized, durable gemmules (Van de Vyver and Willenz, 1991). Gemmulation, especially in species such as *E. muelleri*, is the more significant form of reproduction. Gemmules appear in greatest numbers at the last of the summer when the temperature begins to fall. A gemmule basically consists of a mass of cells called thesocytes that hold large amounts of yolk (Frost, 1991). The thesocyte mass is contained within a tough collagen shell supported by the gemmoscleres (Frost, 1991). In *E. muelleri*, the former adult sponge is reduced to a "mat" of these structures which are embedded into the old skeleton by the time summer ends (Van de Vyver and Willenz, 1974).

Gemmules possess amazing ability to withstand cold temperatures, remaining viable under the ice during the winter months with a minimal rate of respiration (Van de Vyver and Willenz, 1974). The increased photoperiod of Spring and the thawing of the gemmules stimulate the gemmules germination (Van de Vyver and Willenz, 1974). The thesocytes migrate through the micropyle, a small hole in the coat of the gemmule, and begin to organize themselves into a new sponge by producing spicules, differentiating, and dividing in order to form the mature sponge tissues.
Symbiosis

Symbiosis, an intimate relationship between two organisms, is a unique and widespread form of biological adaptation. There are three separate types of symbiosis: mutualism, commensalism, or parasitism (Villee et al., 1984). Mutualism refers to a relationship in which the symbiote, the smaller of the organisms, dependent on the host, and the host, typically the larger organism, non-dependent on the symbiote, both receive some benefit from one another (Villee et al., 1984). Commensalism describes the situation in which the host receives no benefit or detriment while the symbiote increases its biological fitness (Villee et al., 1984). The last possible symbiotic relationship is parasitism. In it, the host is actually harmed while the fitness of the symbiote is again increased. I am going to concentrate on mutualism, the form of symbiosis observed in E. muelleri.

Symbiotic relationships may be seen in all forms of life. The wood-digesting bacteria inside of the intestine of termites and the clown fish living in the tentacles of the sea anemone are both commonly observed symbiotic relationships. A common symbiote that can be found in "cosmopolitan" habitats, both associated with a host and independent, are the small, unicellular algae of the genus Chlorella. Some species are comparable in size to bacteria; therefore, determination of morphological and structural
details of Chlorella are too minute for light microscope resolution (Fott and Nova'kova', 1969). In addition, Chlorella may be cultured similarly (agar slants are often used rather than the liquid media used for most algae). With one exception, Chlorella sp. are the only algal organisms found in symbiosis in freshwater invertebrate hosts, which include sponge, protozoa, flatworms and clams (Frost, 1991). The exception to this is the single sponge genus, Corvomeyenia, that utilize zooxanthellae (Frost, 1991). These Chlorella sp. symbiotes grow, in laboratory culture, to at least twice the size of the free-living and intracellular symbiote forms (Fott and Nova'kova', 1906).

The minute size of Chlorella causes the identification of distinct species to be difficult. In addition, all but one of the individual species of Chlorella that are found in symbiosis with animals such as Hydra viridis, the green hydra, and Spongilla lacustris, a species of freshwater sponge that also possesses symbiotic algae, have not been cultured or identified with respect to their physiological properties (Fott and Nova'kova', 1906).

Chlorella inhabits freshwater sponges within cytoplasmic vacuoles of the sponges' archeocytes and choanocytes at a ratio of one alga per vacuole, while multiplicities of algal vacuoles may be found within a single sponge cell. The photosynthate produced by the
endozoic algae has been shown to be transferred into the sponge cell and may be observed as granules in the cytoplasm of sponge after $^{14}C$ autoradiography treatment (Gilbert and Allen, 1973). There is also a limited amount of intracellular ingestion of Chlorella by the sponge cell. However, this does not occur at a great enough rate to account for a significant part of the sponge's diet. Symbiote digestion seems to occur as a random event leaving photosynthate exchange as the central benefit to the sponge in hosting its symbiotes (Frost and Williamson, 1980). In one species of freshwater sponge, Spongilla lacustris, the photosynthate produced by the algae can account for "50-80% of the growth" of the sponge (Frost and Williamson, 1980). An excellent summation of the sponge-Chlorella relationship was given by Thomas M. Frost (1991):

Algal-invertebrate symbiosis combines autotrophic processes with heterotrophy. In the resulting mixotrophic nutrition, symbiotic algae provide photosynthetically fixed carbon to the invertebrate host, which, in turn supplies nutrients such as nitrogen or phosphorus or carbon dioxide to the algae.

Here Frost has described a textbook model of the mutualism. However, in addition to this, the algae also supplies the host with oxygen and the host's respiration Conversely supplies the algae with carbon-dioxide. The secretion of photosynthetically fixed carbon from the algal cell appears to be a trait shared by all algal symbiotes isolated from their host. When cultured, Chlorella sp. will continue to "leak" fixed carbon to the media. The fact that only
carbohydrates are lost rather than a random mixture of metabolic products further indicates the special mechanism that has evolved in symbiote algae making them highly specific for their environment (Reisser, 1984).

The endozoic algae of freshwater sponges can divide within adult sponge cells, but not in the thesocytes of sponge gemmules. Algae multiply by forming autospores, which are individual daughter-cells contained within the mother-cell's cell wall until their intracellular development is complete and the mother-cell wall lyses. Free-living Chlorella usually reproduce in autospores in numbers in excess of eight, while the symbiotic Chlorella never produce more than four autospores at a time, though the method of this regulation is unknown (Reisser, 1984). The reduced reproduction of symbiote algae insures that the Chlorella will not overrun its host, thus ending the mutualism of the relationship.
Materials and Methods

My project began with the collection of the freshwater sponge, *Ephydatia muelleri*. This species of Porifera was found in Salmon Lake, a Rocky Mountain freshwater lake in Western Montana. The sponges were collected exclusively from the excurrent end of this lake. The regions of the outlet where the sponges were collected bore these characteristics: shallow water, three to four feet deep; crevices between and underneath rocks that kept the majority of the sponge out of direct sunlight; substratum of smooth river rocks that lacked sand and gravel; and a current of water running clear of visible debris. The sponge was removed from its substratum in pieces, the approximate diameter of a quarter (1.5-2.5 cm), with a small knife and was placed in lake-water filled, plastic specimen jars for storage. The specimens were refrigerated at 5-6°C. The sponges were stored one to two days, at most, before dissociation and archeocyte separation.

In order to dissociate sponge cells and separate the archeocytes, a gradient was formed by layering solutions of 4, 6, 9, and 12% Ficoll (Sigma F-4375) dissolved in Calcium-Magnesium Free medium (CMF). The CMF medium consisted of: 6 mM Tris (Sigma, T-1503), 34 mM NaCl (Baker, 1-3632), 1.34 mM KCl (Sigma, P-4505), 1.38 mM glucose (Sigma, G-8270), 1.07 mM NaHCO3 (Sigma, S-8875), 0.7 mM (10)
K2HPO4 (Baker's, 2749), and 0.25 mM EDTA (Amachem, ES-95). Each of these layers had a volume of 3 ml. The Ficoll was driven into solution by agitating the solution with a magnetic stir-bar while the Ficoll solution beaker sat in a bath of warm tap water. The Ficoll solutions were then successively layered, starting with the addition of the most concentrated solution, 12% Ficoll, to a sterile 15 ml centrifuge tube and then slowly adding the second most concentrated solution, 9% Ficoll, over the top of the first layer by running the 9% solution along the side of the centrifuge tube with a Pasture pipette. Solutions of 6% and 4% were then layered in the same manner as the 9% solution. The completed gradient was spun, prior to the addition of cells, at 5000xg for 5 min in a Sorvall Superspeed RC-2B centrifuge using an SS-34 rotor in order to smooth out the gradient.

Next, the cell suspension was prepared by soaking approximately 5 g (wet-weight) of finely minced sponge that was free of dirt and debris, in 5 ml cold CMF for 5 min. The CMF medium, the same mentioned above, was prepared in quantities of 250 ml. These reagents were dissolved in twice distilled, sterilized (Castle autoclave) water and then taken to volume. The pH, which was not adjusted to 7-8, as proscribed by De Sutter and Buscema (1977), was tested with pH indicator strips at slightly above 8. After the 5 min incubation, the tubes containing
the CMF-sponge combination were vortexed with a single setting Fisher Scientific Vortex for 3 x 10 second impulses. Then, the suspension was repeatedly trituated with broken tip Pasteur pipette (first with a wide, jagged bore and then with a narrower bore) and vortexed again as outlined above.

After the last vortexing, the larger particles were allowed to settle, then 2 ml of the cell suspension were layered on top of each of the Ficoll gradients. These gradients were then spun in the centrifuge listed above at 5000xg for seven minutes with the Auto-brake turned off. The tubes were then removed. The band that contained the purified archeocytes, at the 9-12% interface, was pipetted out being so as not to remove any other part of the suspension. This suspension was then placed in a cold, sterile tissue homogenizer (1 ml capacity).

The suspension was gently homogenized by hand in the afore mentioned tissue homogenizer. The pestle was twirled and simultaneously pushed and pulled in and out of the homogenizer tube at a rate that allowed the fluid to pass between the pestle and the glass with out forming bubbles under the pestle. This was done in order that the archeocytes would be opened without also rupturing the algal cells that were contained within the sponge cells. This suspension was then added to 25 ml of sterile Alga-Gro (Carolina Biological Supply Company) in a sterile, covered
dish, and a cell count was made with a Spencer Bright-line Hemocytometer. The Alga-Gro was made by bringing a 10 ml vial of concentrated Alga-Gro solution to a volume of 500 ml with double distilled water and then autoclaving this solution in a screw-capped bottle for 15 min at 20 psi and 121°C with a Castle sterilizer.

A second algal culture was made of algae associated externally with the sponge. About 5 g of sponge tissue were vortexed in a plastic, 15 ml centrifuge tube with 5 ml sterile double distilled water. The green supernatant of this suspension was pipetted from the settled sponge material and then brought to a volume of 25 ml with sterile Alga-Gro in a sterile, covered dish as well. A cell count was then made in the same manner as for the prior culture. These two algal cultures were the stock cultures which were used for subsequent characterizations and experiments.

Growth rates were determined by starting a new culture from the stock cultures diluted to the cell density of the original cell count of the stock solution. Daily cell counts were made for a period of two weeks. The stock cultures had been maintained for 4 months before subculturing for growth rate determination.

Gemmules from *E. muelleri* were collected from the same area as the sponge utilized for the algae isolation. These gemmules were kept refrigerated (5°C) until a week before their use in December, when they were frozen at (13)
-20°C and then thawed slowly (5°) for use. The gemmules were first treated with a 1% hydrogen peroxide solution at room temperature for 5 min. Then the gemmules were washed five times in distilled water (2 min each wash) (Clifford, 1991). The gemmules were then placed on coverslips that had been submerged in 10 cm plastic culture dishes filled with 30 ml lake water. The lake water had been taken from the same collection site as the gemmules and sponge tissue on 10/31/93 and stored at 5-6°C. Before use it was filtered through a 0.45 μm Acrodisc. Then, roughly four days after plating, the gemmules germinated.

Two runs were made. In the first run two clean culture plates were submerged with the plate of germinated gemmules in a tank partially filled with distilled water. On each of the new plates, half of the gemmules were transplanted, keeping them submerged during transfer in order to avoid disrupting their fragile structure. These plates were then removed from the tank, and the distilled-water was gently aspirated from them. The water was then replaced with a volume of 28 ml lake-water, filtered as above and 2 ml algae suspension (1.47 x 10^7 cell concentration) in the first plate and 2 ml sterile Alga-Gro in the control plate. The second run was prepared in a similar fashion with the exception that three different plates were run following germination. The first two plates were prepared as before except they had a total volume of 40 ml rather
than 30 ml. The third dish, which also had 40 ml total volume, had 2 ml of algae culture that had been filtered through 0.45 μm pore Acrodisc rather than sterile Alga-gro. These plates were left at room temperature for a week. At the end of this period two gemmules were removed from each dish for photography.

Photography was done by using a Nikon Optiphot microscope with a 35 mm camera mount. Photographs were taken of germinated gemmules by first inverting the coverslip that the gemmule had adhered to and placing it on a deep-well glass slide with several drops of distilled water. Algal samples were mounted on standard glass slides. The samples were then photographed using 40x and 100x objective lenses. Both brightfield microscopy and phase-contrast microscopy were used to view specimens.
RESULTS

Algal Cultures

After centrifugation of the cell suspension into the Ficoll gradient, milky-white bands at all three interfaces were observed. The top layer, which lacked Ficoll, remained green and the bottom of the centrifuge tube had a large, dark-green pellet. The band at the 9%-12% interface contained a population of archeocytes (Fig. 1a-b) which contained the endozoic algae, *Chlorella sp.*

The algal cell culture taken from the archeocytes showed fairly steady linear growth which leveled off after 11 days (Fig. 8). This culture consisted of *Chlorella sp.* (Fig. 2) as well as other algal cells of both filamentous and spherical appearances (not classified) (Fig. 3). On the sixth day, the algal cell-wash culture population plummeted below the threshold for an accurate count, less than 200 cells. In the place of algae were large flagellated protozoans. The algal cell populations of this culture did not recover during the two week study.

Algal Uptake

When control sponges (untreated sponge and sponge treated with filtered extract) and the experimental sponges
(grown in the presence of algae) were examined after 7 days, it was observed that both experimental and control sponges contained symbiotic algae (Fig. 4-7). Not only were there no noticeable differences in the number of endozoic algae between the control and the experimental group, but there were no detectable differences in intracellular structure of body form between the two populations of developing sponge.
Fig. 1a (A) archeocyte (1000x) containing multiple vacuoles, many containing (C) Chlorella sp.

Fig. 1b Wide shot (400x) of fig 1a showing (B) another archeocyte.
Fig. 2 *Chlorella* sp. cells (1000x) isolated from archeocytes.

Fig. 3 Other forms of algae in culture (1000x) (from left to right): long multicellular filaments, large spheres with many chloroplasts, and two rods with little pigment and a division at 1/3 of their length.
Fig. 4 Gemmule (yellow sphere) after germinating. Sponge material is the dark crescent around the gemmule and the thin white film surrounding the gemmule.

Fig. 5 (A) Archeocytes photographed at 1000x phase contrast. (C) Chlorella sp. may be visualized even though this was taken from a control plate.
Fig. 6 (A) Archeocyte at 1000x phase contrast from algae cultured gemmules. (C) Chlorella sp. may be seen in vacuoles inside the cell.

Fig. 7 Photograph of the periphery of a (G) gemmule with two (A) archeocytes free of the cell mass. these archeocytes also contain Chlorella sp. in vacuoles although they are from the control gemmule dish. (400x)
FIG. 8  GROWTH CHART OF ALGAL CULTURE
Discussion and Conclusions

The isolation of an archeocyte population from a crude dissociated sponge suspension was successful. When the cellular suspension from the 9-12% interface was observed by light microscopy, it was found to be a highly pure fraction of vacuolated archeocytes (free from any observable contaminants). From this fraction, a culture consisting mostly of the algal symbiotes with some other algal forms was established and maintained in vitro for a period of five months. This stock culture was successfully subcultured, although the ratio of *Chlorella* sp. to other algal forms decreased with time in both the stock cultures and subcultures (data not shown). It is possible that the mixture was contaminated by other algal forms when the band from the 9-12% interface was aspirated off or other algal forms may have entered the archeocyte culture because they had been engulfed previously by an archeocyte for food.

The cell wash culture, however, did not survive in vitro, being overgrown (or consumed) by a population of flagellates. The algal population within the cell wash culture did remain viable for a sufficient time to allow some basic comparisons between it and the archeocyte culture. The cell-wash culture contained a greater variety of algae and far fewer *Chlorella* than the archeocyte culture.
did. The cell wash culture also contained a variety of protozoans, while none were observed in the archeocyte culture. There is a possibility that the algae obtained in the cell-wash culture were not associated with the sponge, rather, they had been pulled into the sponge to be used for nutrition prior to collection. According to work done by Frost and Williamson (1980) rarely are any organisms found associated with the sponge extracellularly. This is thought to be a result of antibiotics that the sponge secretes into its surrounding environment. The cell suspension obtained was very concentrated, indicating the presence of far more algae than the observations of Frost and Williamson suggested should have been present.

The ultimate objective of observing uptake of symbiotic Chlorella sp. was not achieved. The lack of uptake or morphological change may be rooted in several factors. First, the enlarged size of algae due to culture conditions may have hindered the sponges ability take in the Chlorella sp. Second, the white appearance of the erupted gemmules was misleading to the fact that they already contained a large number of archeocytes. The fact that the symbiotes were already present may have hindered the uptake of algae. Additionally there may not have been enough cultured algae present in the gemmule culture to achieve observable results. Lastly, the short incubation period may have clouded results as well. Uptake of the Chlorella sp. by
E. muelleri in laboratory conditions may still be possible to observe, but not in the short incubation period used in this study.

The short incubation period was, in my opinion, this study's greatest drawback. Although the gemmules germinated and remained viable for the duration of each run, the fact that freshwater sponges require sensitive culturing conditions, namely cooler than room temperature water temperature of 10-15°C (which I was unable to control), made E. muelleri difficult to keep viable over long periods of time. In previous observations the sponges consistently deteriorated rapidly after 7 days.

In conclusion, with continued success in archeocyte isolation and algae culturing, the analysis of symbiotic algae uptake maybe possible if some or all of these conditions are met: acquisition of an aposymbiotic strain of E. muelleri; culturing of the symbiotic Chlorella sp. at its naturally occurring size; and prolonged laboratory culturing of the freshwater sponge E. muelleri.
Works Cited


