Community structure: environmental biofilms and purple non-sulfur bacteria

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Mini-project #1: To assess the diversity in the members of biofilm and planktonic communities from an enrichment from Eel Pond.

Introduction

While free-swimming, planktonic cells have often been the choice of cultures for microbiologists to study it is thought that the majority of bacteria in the environment are associated in bacterial communities called biofilms. A biofilm is a surface-attached community that is usually embedded in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, and nucleic acids (3-5). A hallmark trait of biofilms is increased resistance to antimicrobial agents compared to the resistance of free-swimming organisms (1).

While communities of biofilm bacteria attached to surfaces are thought to be the dominant form of bacteria in the environment, little is understood about the community structure of environmental biofilms. Most research on biofilms has focused on single-species biofilms and at most dual-species biofilms, with the exception of activated sludge biofilms in wastewater treatment plants. Biofilms in the environment may be involved in elemental cycles as well as the accumulation of pollutants such as heavy metals (2) and it is important to identify what organisms in an environment are present in biofilms. Little is know as to what organisms are present in environmental biofilms and how this community differs from the planktonic community. This study sought to identify community members in the biofilm and planktonic state over time in enrichments and compare these communities to the community present in the initial inoculum.

Materials and Methods

Culture conditions

Seawater from Eel Pond (15 mL) was inoculated into 4 plastic 250 mL centrifuge bottles containing 150 mL SW-P media. SW-P media was composed of (per liter): 1L seawater base media, 1 mL trace nutrients, 1 mL SL-12 vitamin solution, and 10 mM sodium pyruvate and the pH was adjusted to 7.0. The bottles were placed at 30°C with shaking at 100 rpm. Duplicate planktonic and biofilm samples were harvested after 3 and 6 days. Planktonic samples were harvested by removing 30 mL of the liquid in the bottles. Biofilm samples were collected by removing all the liquid from the bottles and draining the bottles upside down for 2 min. New media was then added and the bottles were sonicated for 5 min and vortexed for 2 min and 30 mL of the liquid was collected.

DNA extraction

Cell cultures from the biofilm and planktonic cultures were concentrated by either centrifuging the sample or by filtering onto a 0.2 μm Millipore isopore filter if a pellet could not be formed. Water from Eel Pond (300 mL) was filtered using a 0.2 μm nylon

filter in order to collect enough cells to extract an appreciable amount of DNA to use as an indicator of the diversity in the inoculum. The filter or the cell pellet was then added to a bead beat tube from the MoBio UltraClean soil DNA kit and bead beat for 5-10 sec and freeze-thawed 5 times. The DNA was extracted using the manufacturers protocol as modified by Brian Wade.

Assessing community diversity

Community diversity was analyzed with terminal restriction fragment length polymorphism (T-RFLP) using the 16s rRNA gene. The 16s rRNA for each time point was amplified from the community DNA using a fluorescently-labled 8F primer and 1492R primer. The sequence for 8F was (5' to 3'): agagtttgatcmtggctcag. The sequence of 1492R was (5' to 3'): ggttaccttgttacgactt. The PCR products were cleaned using Microcon filters (Millipore). The 16s rRNA of each time point was digested with HhaI for 3 h at 37°C and the enzyme was inactivated at 65°C for 10 min. The samples were ethanol-precipitated and allowed to air-dry overnight at room temperature in the dark. Originally, there were duplicate samples for both the biofilm and planktonic samples for each time point, but due to mishaps during processing the duplicate samples for day 6 were lost. T-RFLP analysis was done at Michigan State University.

Identifying community members

To identify members of the biofilm and planktonic communities, a clone library was made using DNA from the day 3 sample. The 16s rRNA of each sample was amplified using the 8F and 1492R primers. Ligation and transformation was done using the TOPO TA Cloning kit (Invitrogen) and samples were plated onto LB plates containing 100 μg/mL ampicillin and 1.6 mg of X-gal (40 μL of 40 mg/mL) and grown overnight at 37°C. White colonies (48 each) were picked from the duplicate biofilm and planktonic samples into 96-well microtitre plate containing LB and 100 μg/mL ampicillin and grown overnight at 37°C. These plates were centrifuged and resuspended in water and freeze-thawed 5 times and centrifuged again to remove cellular debris to the bottom of the well. Clone sequences were amplified using the M13 forward and reverse primer. Only half of the clones were amplified, 24 from each of the 4 samples. Clone sequences were cleaned using the XO Sap it at 37°C for 30 min and 80°C for 15 min and then sequenced at Michigan State University.

Results

Microscopic examination

Examination of the samples using a microscope showed that there was diversity in the morphologies in the bacteria present in the samples. Both day 3 planktonic cultures had rods and cocci, both motile and non-motile. There were some dinaflagellates present as well. The same morphologies were present in the biofilm cultures as well, but no dinaflagellates were present. Day 6 planktonic cultures were mostly rods (motile and

non-motile), with some spirillum (Fig. 1A,B). The biofilm cultures had cocci as well (Fig. 1C,D). Clumps of bacteria were present in both the planktonic and biofilm cultures.

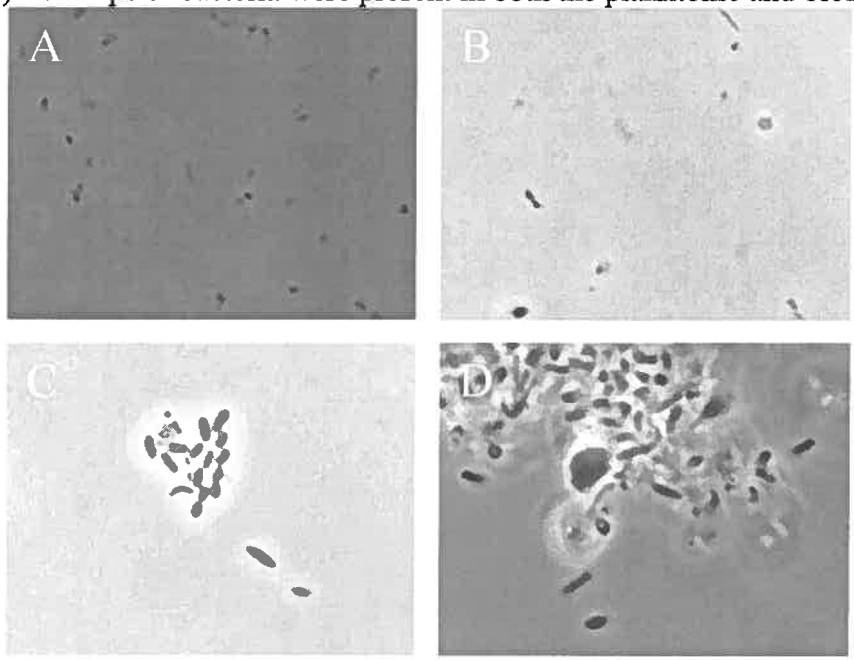
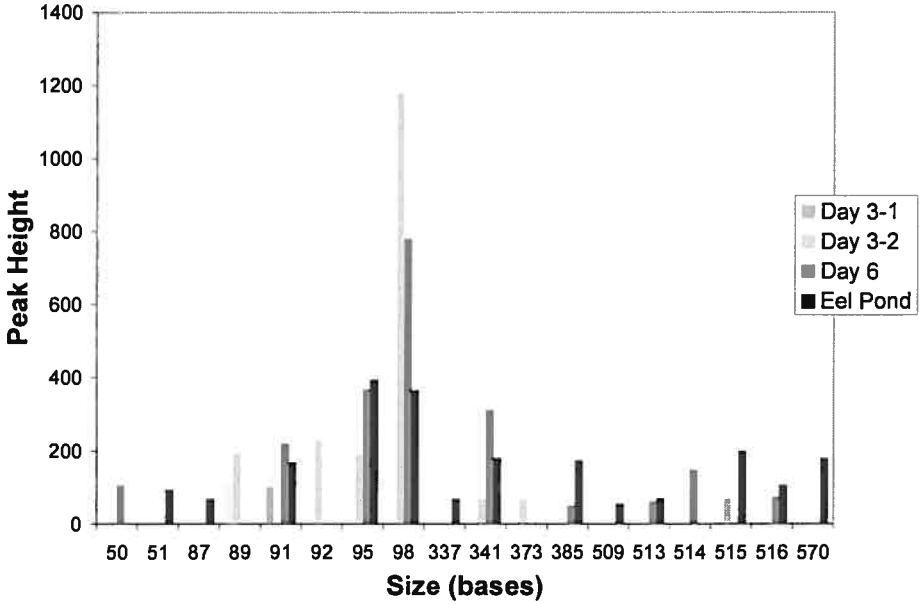


Figure 1: Morphologies present in day 6 biofilm and planktonic communities. A) and B) planktonic samples. C) and D) biofilm samples. Micrographs are taken with 100X magnification.

T-RFLP

There was little difference between the planktonic enrichment T-RFLP pattern and that found from Eel Pond water. Peaks at 50-51, 89, 91-92, 95, 98, 341, 513-515 bases are present in both the planktonic culture and the Eel Pond sample (Fig. 2). The TAP-TRFLP program from RDP (http://rdp.cme.msu.edu/html/) was used to find bacteria that could have digested fragments at the same base size as major peaks in the samples. Peaks at 95 and 98 bases could be members of the *Cytophaga, Desulfovibrio, Campylobacter*, or *Desulfobacterium* genera. The planktonic sample for day 3-1 has only 2 small peaks, which are probably due to a low DNA concentration present in the digested sample and this sample can be ignored. There appears to be little change in the diversity in the planktonic community between day 3 and day 6.

There appeared to be more members in the biofilm community as there were more peaks present at different fragments sizes in the T-RFLP results (Fig. 3). The biofilm samples had the same peaks as the planktonic samples at 50-51, 89, 95, 98, 341, 513-515 bases. However, there were also peaks at 54, 57, 72, 77, 205, 385, and 563 bases. The peaks at 89 bases could be due to members from *Azospirillum, Capnocytophaga*, or *Haliscomenobacter*. The peak at 54 bases could be due to *Haloanaerobic, Craurococcus*, or *Sporohalobacter*. The biofilm samples are also fairly similar to the community found in the sample from Eel Pond water. The diversity in the biofilm samples seems to decreases between day 3 and day 6 in that the number and distribution



of peaks decrease over time. The peaks at 54, 72, 98, 341, and 385 bases are present at day 3 but disappear by day 6.

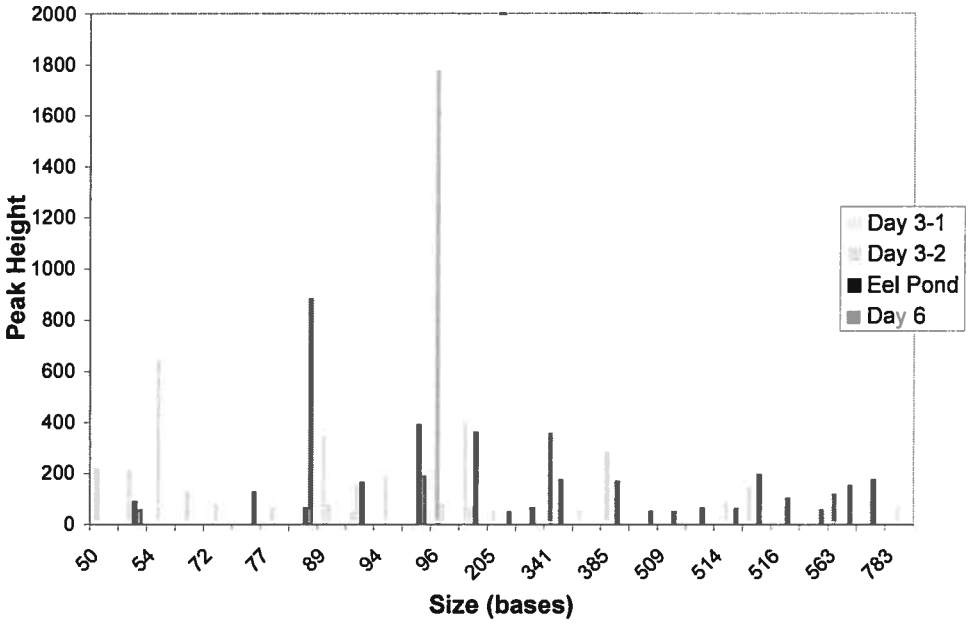


Figure 2: Significant T-RFLP peaks found in the planktonic community after days 3 and 6 and as compared to the inoculum water from Eel Pond.

Figure 3: Significant T-RFLP peaks found in the planktonic community after days 3 and 6 and as compared to the inoculum water from Eel Pond.

Sequencing results

From the clones that were sequenced for the biofilm and planktonic samples there appeared to be no difference between the two samples in the distribution of what the clones were most similar to. There were a total of 58 returned sequences from the sequencing facility. The Sequence match tool from RDP was used to identify the sequence of the clones. There were 12 clones that matched as the Gamma-Proteobacteria *Alteromonas macleodii* and 8 clones that matched as the Gamma-Proteobacteria *Oceanospirillum* in the *marinomonas* assemblage. Both *Alteromonas* and *Oceanospirillum* are Gram negative and grow in seawater. There were also 11 clones that matched in the *Cytophaga* family and these were most closely related to *Cytophaga uliginosa, Cytophaga marinoflava, Flavobacterium gondwanense,* and *Flavobacterium salegens*. There were 20 clones that matched with the *Campylobacter* family (Fig. 4). The clones that were in the *Campylobacter* family were most closely related to *Campylobacter concisus*, *Arcobacter nitrofigilis*, and *Arcobacter butzleri*. *Campylobacter* can cause enteritis in humans and one route of infection is by eating clams and shellfish, which are present in Eel Pond. For these *Campylobacter* clones, it is likely that there were only three different strains since there were three distinct clusters of clone sequences. Since there were *Campylobacter* and *Cytophaga* strains in the clone library, the T-RFLP fragments at 95 and 98 bases were most likely due to these species and not from *Desulfovibrio* and *Desulfobacterium*. The remaining 7 sequences matched as the alpha-Proteobacteria *Rhodobacter*, Gamma-Proteobacteria as *Pseualteromonas* and *Pseudomonas*, delta-Proteobacteria as *Bdellovibrio*, and as the Gram positive *Fusibacter*.

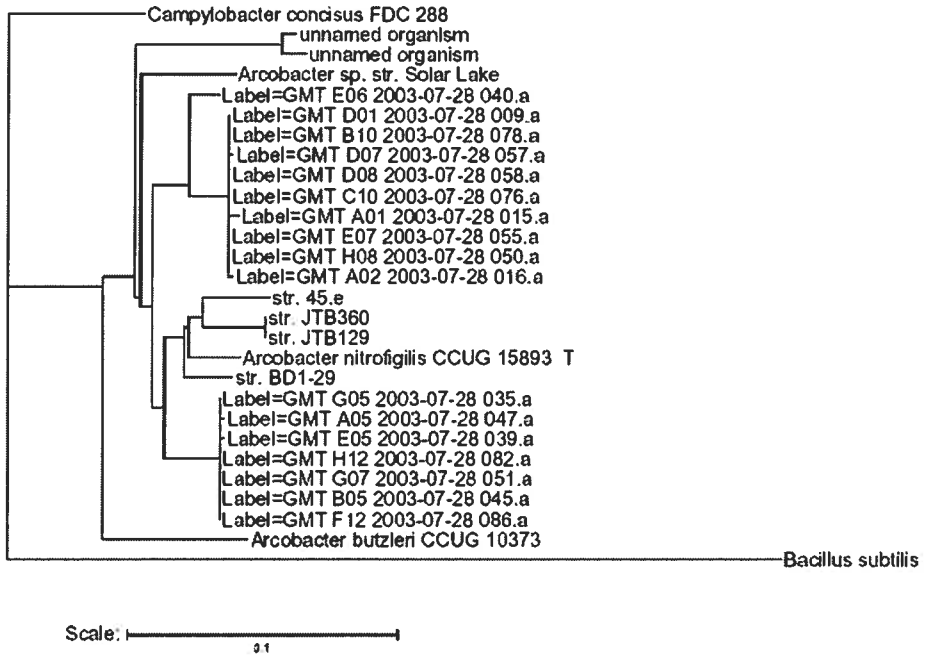


Figure 4: Phylogenetic tree composed of clones matching closest to the family *Campylobacteraceae* and specifically *Campylobacter* and *Arcobacter*. Biofilm and planktonic clones from this study are labeled as "GMT X##." Planktonic samples range from 01-06 and biofilm samples range from 07-12.

Conclusions and future directions

The community diversity found in the biofilm and planktonic samples was similar to that found in the Eel Pond water used for the inoculum of the samples. There appeared to be little change over time in the planktonic community. The diversity in the biofilm community was greater than that of the planktonic sample, but appeared to decrease with time. The T-RFLP and clone library results show that there are likely *Cytophaga* and *Campylobacter* present in the enrichment. Sequencing results also indicated the presence of *Alteromonas macleodii* and *Oceanospirillum,* which are both found in the marine environment.

In the future if this experiment is repeated on a longer timescale, then the cultures should be resupplemented with nutrients. It appeared that the total number of bacteria was going down from microscopic evaluation from day 3 to day 6 and this could be from nutrient limitation. In addition, it would be good to run multiple digestions of the T-RFLP samples using different enzymes to get more information on the potential identity of the peaks present in the sample.

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Mini-Project #2: To identify the members of the purple satellite formed in my purple sulfur bacteria agar shake tube and to identify why they form interactions in the purple sulfur bacteria media.

Introduction

Examination of my purple sulfur bacteria (PSB) agar shake tube showed that there were satellite colonies of large white bacteria surrounded by many smaller purple colonies (Fig. 5). There was an increase in the density of the purple bacteria around the white colonies as compared to further away from the white colonies. No white bacteria were observed alone. Why should a white, non-phototrophic bacteria be present in my PSB shake tube? It appeared that the white and purple bacteria were assisting each other to live in the PSB shake tube, but the exact interaction and the identities of these bacteria were unknown.

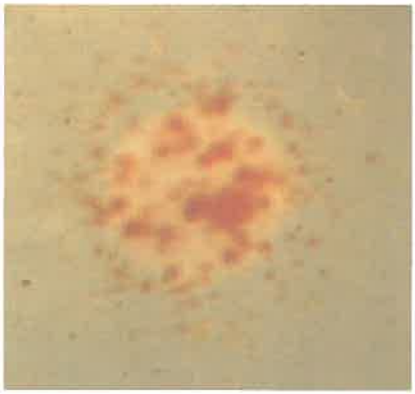

Figure 5: Micrograph of purple satellite taken from the PSB agar shake tube. Note the white colony surrounded by many purple colonies. This micrograph was taken using the dissecting microscope.

Materials and Methods

Culture conditions

Original cultures of PSB were inoculated from mat samples from Sippewissett marsh into Pfennig bottles containing PSB media and incubated at room temperature next to 40W lightbulbs. A serial dilution of shake tubes was made using 1 mL from this culture and added to 6 mL PSB media and 3 mL agar. The headspace in these tubes was composed of N_2 and CO_2. Single colonies or mixtures from the shake tubes were removed and resuspended in PSB media to transfer to new liquid PSB tubes, new shake tubes, and to plate out onto many different agar plates for single colony isolation.

Carbon source growth curves

The carbon sources that the white strains could utilize were tested using a basal media. This media was composed of (per liter): 1 L seawater base media, 1 mL trace nutrients, and 1 mL SL-12 vitamin solution and the final pH of the media was adjusted to ~7.0. The following carbon sources were added to aliquots of the basal media with a final concentration of 10 mM: glucose, galactose, fumarate, butyrate, ethanol, p-hydroxybenzoate, succinate, and acetate. Growth in these different media types was assayed in a 96-well microtitre plate containing duplicates of each media type for the strains GT1-GT5. Growth was measured using optical density at 655 nm. This wavelength was chosen because there was a minimum in absorbance from 500-700 nm (Varian Cary Bio 50 UV-visible spectrometer) and the only filter the microtitre plate reader (BioRad) that was in this region was at 655 nm.

Sequencing

A single colony of isolated strains was added to 50 μL sterile water and freeze-thawed 5 times. The cells were heat-popped to release the DNA at 96°C for 10 min and then centrifuged to concentrate cellular debris at the bottom of the vial. The 16s rRNA gene was amplified using the 8F and 1492R primers. The sequence for 8F was (5' to 3'): agagtttgatcmtggctcag. The sequence of 1492R was (5' to 3'): ggttaccttgttacgactt. The PCR products were cleaned using Microcon filters (Millipore) and sent to be sequenced at Michigan State University. Sequences were identified using the Ribosomal Database Project website (http://rdp.cme.msu.edu/cgis/seq_match.cgi?su=SSU) and by doing BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/).

White Supernatant study

This experiment was to see whether the supernatant from the white strains was promoting growth of the purple strains. The white strains GT1-GT4 were grown up in SW media supplemented with 10 mM succinate and then centrifuged. The supernatant was sterile filtered using a 0.2 μm syringe filter. Two milliliters of PSB media was supplemented with 1 mL of supernatant. The media was inoculated with a suspension of GT7 and GT9 and the headspace was exchanged to N_2 and CO_2. The tubes were incubated at room temperature in front of 40W lightbulbs.

SW-PNS media study

A basal media for growth of purple non-sulfur bacteria was designed. SW-PNS media was composed of (per liter): 970 mL seawater base media, 1 mL trace nutrients, 1 mL SL-12 vitamin solution, and 30 mL 1M $NaHCO_3$ and the final pH of the media was adjusted to ~7.4. The basal media was autoclaved, and then cooled under a stream of N_2 and CO_2 surrounded by ice to keep the media anaerobic. Then the vitamins, trace nutrients, and buffer were added and the media was dispensed into and completely filled screwtop vials.

Results

Isolation results

Plating out a mixture of the bacteria present in the purple satellite along with the white colony in the middle yielded many different isolates on different media types from both aerobic and anaerobic growth. Two different morphologies of white colonies were observed: a larger white round colony and a smaller clear-white round colony. These were present on aerobic SWC and PNS-succinate plates. White colonies were also present on an aerobic SW-POB plate and anaerobically on SWC. Purple colonies were observed on anaerobic plates of PNS-ethanol, PNS-succinate, SWC, GYT, and SW-POB as well as aerobically on SW-POB.

Five white strains (GT1-5) and 2 purple strains (GT7 and GT9) were primarily used from the isolations (Table 1). From microscopic evaluations, I began to suspect that GT1 and GT3 were the same and GT2 and GT4 were the same. I isolated GT1 and GT3 from different agar plates, but both formed large round white colonies on agar plates. Both were also short, fat rods (Fig. 6). GT2 and GT4 also appeared as similar colony types on agar plates, as round, clear-white colonies and were thin rods. GT7 and GT9 were purple colonies and were both rods.

Table 1: Description of strains isolated and used in this study

Strain	Media isolated from	Colony Morphology	Morphology	An/aerobic
GT1	SWC	Round, large white	Short, fat rods	Aerobic
GT2	SWC	Round, large white	Thinner rods	Aerobic
GT3	PNS-succinate	Round, clear-white	Short, fat rods	Aerobic
GT4	PNS-succinate	Round, clear-white	Thinner rods	Aerobic
GT5	SW-POB	Round, white	Thinner rods	Aerobic
GT7	SWC	Round, purple	Rods	Anaerobic
GT9	PNS-ethanol	Round, purple	Rods	Anaerobic

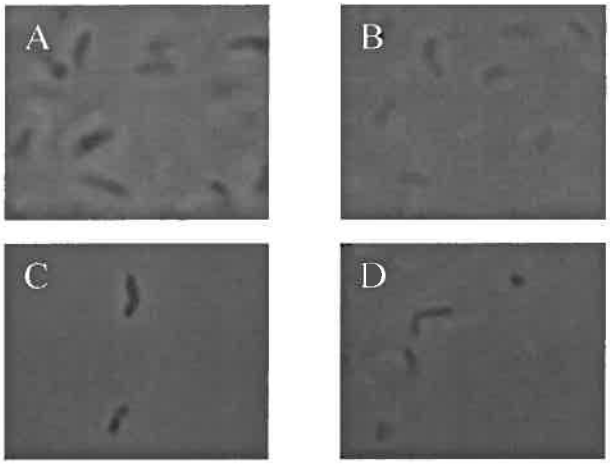

Figure 6: Micrographs showing the morphologies of GT1-4. A) strain GT1. B) strain GT2. C) strain GT3. D) strain GT4. Micrographs were taken with 100X magnification.

Carbon source preferences of white colonies

Growth of GT1-5 in the presence of different carbon sources was monitored over three days. All strains grew well on glucose, fumarate, and succinate with generation times of 1-3 hours (Table 2). GT2 and GT4 were capable of growth on glucose, galactose, succinate, and fumarate. GT1 and GT3 were capable of growth on these and acetate as well. GT5 had a different profile and did not grow on galactose, but was capable of growth on p-hydroxybenzoate and ethanol. The capability of growth on acetate seems to also be another factor in grouping GT2 and GT4 together and GT1 and GT3 together since GT1 and GT3 are capable of growing on acetate.

Table 2: Generation times (in hours) of GT1-5 on different carbon sources

	GT1	GT2	GT3	GT4	GT5
Glucose	0.71	0.93	0.83	0.76	1.67
Galactose	2.09	1.12	2.41	1.43	
Succinate	3.44	1.36	2.26	4.34	1.03
Fumarate	2.76	1.14	2.08	1.68	0.87
Acetate	5.54		2.89		1.14
p-hydroxybenzoate					1.28
Ethanol					3.16

Sequencing the white strains

The 16s rRNA of the white strains GT1-5 was amplified and purified and sent to be sequenced, however only GT1 and GT2 were able to be sequenced. It was found that both were *Vibrio* sp. with their closest relatives being V. *parahaemolyticus*, *V. natriegens, V. anguillarum, V. alginolyticus, Listonia anguillarum, V. campbelli, V. ordalii,* and *V. aestrianus*. GT1 and GT2 have different closest neighbors in a phylogenic tree (Fig. 7) and are most likely distinct strains.

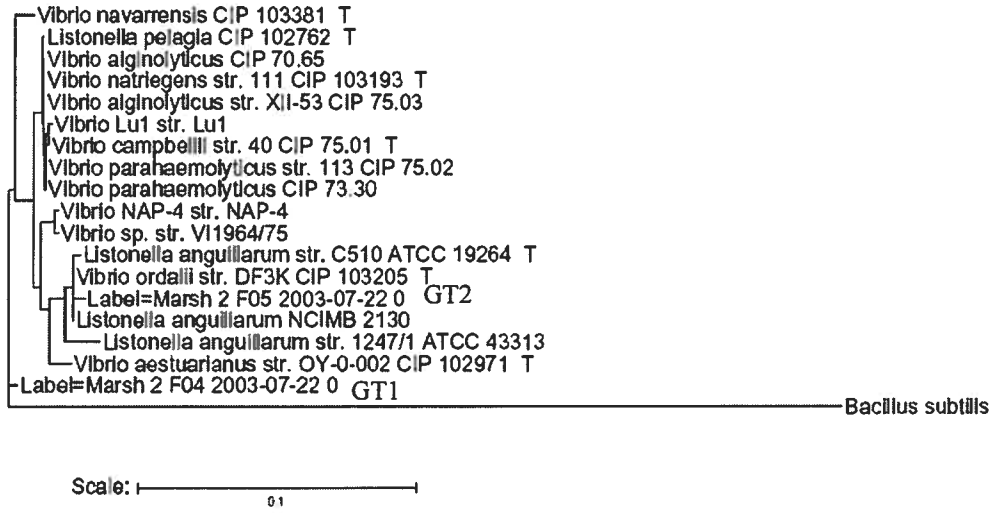

Figure 7: Phylogenetic tree showing the similarity between GT1 (Marsh 2 F04) and GT2 (Marsh 2 F05) and the genera *Vibrio*.

Investigating the purple bacteria: are they really purple sulfur bacteria?

Wavelength scans of purples

A wavelength scan was performed on colonies scraped from agar plates and placed between a microscope slide and coverslip and it was found that both GT7 and GT9 had absorbance at 804 and 850/855 nm. GT7 had absorbance at 855 nm while GT9 had an absorbance peak at 850 nm. This absorbance profile indicates the presence of bacteriochlorophyll a. Bacteriochlorophyll a can be present in both purple sulfur and purple non-sulfur bacteria (PNS).

Growth on PSB media

Once isolated, growth of GT7 and GT9 in PSB media anaerobically with light was monitored. There was no appreciable growth of these strains in PSB media. Since this was in 5 mL of media and one colony was used to inoculate the tubes, perhaps this was too much media to see growth quickly. GT7 and GT9 were then inoculated into only 2 mL PSB media and 1 mL of sterile filtered supernatant from GT1-4 as well as a control of 3 mL PSB media. It was found after 48 h that GT7 and GT9 grown with supernatant from GT2 and GT4 were turbid and had produced a pink pigment (Fig. 8). GT9 grew on GT3 supernatant as well, but did not produce any pigment. There was no growth of GT7 and GT9 in only the PSB media. Something in the supernatant from the white strains must be beneficial for the growth of GT7 and GT9 in PSB media. It is possible that the purple strains could be utilizing leftover succinate found in the growth media for the

white strains or they could be utilizing compounds excreted by the white strains. Supernatant from GT1-4 was analyzed for acetate using high performance liquid chromatography (HPLC), however acetate was not found while several other unidentified compounds were.

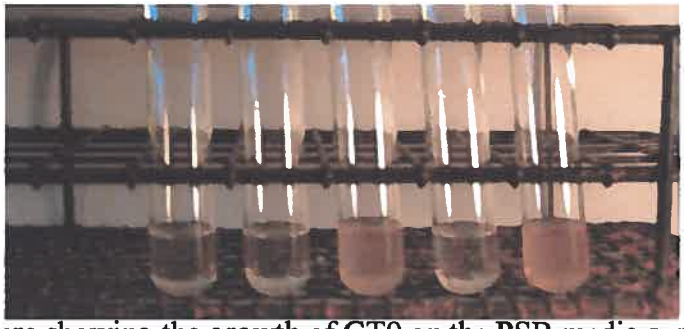

Figure 8: Picture showing the growth of GT9 on the PSB media supplemented with supernatant from the white strains. Additions from left to right: no addition, supernatant from GT1, supernatant from GT2, supernatant from GT3, supernatant from GT4.

At this point, it was suspect whether GT7 and GT9 were PSB. They did not grow well on PSB media and seemed to prefer having a carbon source present during photosynthesis. PNS also can perform anoxygenic photosynthesis and tend to do so as photoheterotrophs instead of photolithotrophically as PSB does. A media was designed to promote growth of purple non-sulfur bacteria (SW-PNS media). Tubes of this media were supplemented with 10 mM succinate, acetate, thiosulfate, thiosulfate + acetate, and thiosulfate + succinate. GT7 and GT9 were capable of growth on all of these substrates. Microscopic evaluation of GT7 and GT9 grown on thiosulfate showed that the cells were dark and did not accumulate sulfur either intracellularly or extracellularly (Fig. 9). Accumulation of sulfur granules is typical of PSB, and while this is not an absolute characteristic of PSB most PSB do accumulate sulfur granules while most PNS do not (6, Jane Gibson- personal communication). In addition, GT7 and GT9 were capable of aerobic growth, which is another indicator that they could be PNS.

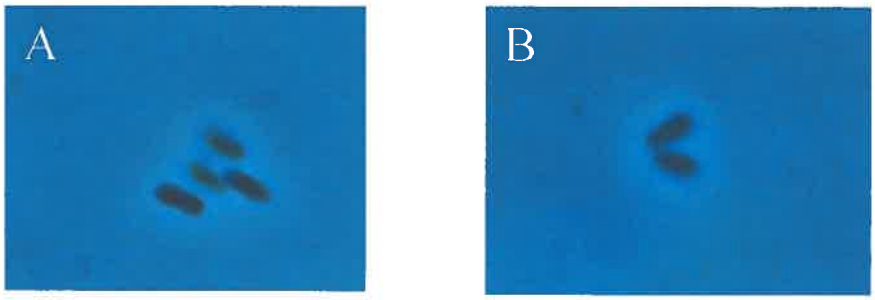

Figure 9: Micrographs of purple strains after growth with thiosulfate. A) GT7. B) GT9. These dark cells are not accumulating sulfur intracellularly and could indicate that GT7 and GT9 are purple non-sulfur bacteria. Micrographs were taken at 100X magnification.

Conclusions and future directions

The individual components of the purple satellites were identified and examined separately in this study, however it is still not clear how these bacteria interacted in the PSB shake tube. The white strains appear to be *Vibrio* sp. and are aerobic heterotrophs. The purple strains are not PSB, but are probably instead PNS bacteria. These strains grow without each other on other media types and it was impossible to recreate the purple satellite phenomenon on anything but PSB media. It could be that the purple satellites formed in the PSB media since neither the white or the purple strains were capable of growth by themselves on the PSB media. The initial colonization of both in the PSB enrichment could have been due to the organic matter present in the initial inoculum from the Sippewissett marsh and some of this could have been carried over into the shake tube. The white and purple strains could also have been living on the agar found in the PSB shake tube, although none of the isolated strains were capable of growth on agar or agarose plates.

Further experiments could be done to determine what compounds the purple and white strains exchange to stimulate their growth on the PSB media. These compounds could be organic compounds. Further HPLC studies could be done on the supernatant of the white and purple cultures to identify compounds present using compounds other than acetate as standards such as succinate, glucose, or fumarate. Other analytical techniques could be used to identify the products such as gas chromatography-mass spectroscopy.

Another experiment that I would have liked to have done would have been to run ARDRA on the 16s rRNA from the white and purple strains. DNA was isolated and 16s rRNA was amplified, but I could not get the 16s rRNA to digest and yield a banding pattern for the white strains. My negative and positive controls were fine, and this problem persisted for different restriction enzymes and PCR products (cleaned and uncleaned). It would have been nice to show that GT1 and GT3 were the same and that GT2 and GT4 were the same using this technique.

Acknowledgements

I am grateful for the help with PSB and PNS from Carrie Harwood, Jane Gibson, Adam Martiny, and Faith Harrison.

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