A Two Part Investigation of Salt Pond Water Column:

Enrichment of Iron and Manganese Reducing Bacteria from the Pink Layer of Salt Pond

and

Community Analysis of Salt Pond Water Column DNA

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Abstract

Salt Pond (Woods Hole, MA) is a seasonally stratified pond with an oxic-anoxic transition zone (OATZ) occurring between 3.25 and 3.5 meters depth. At 3.5 meters depth, the water in this zone appears pink and previous characterization reveals that soluble iron two peaks in this zone. Limnological profiles of oxygen, pH, salinity, and temperature were obtained and nutrient and metal analyses are currently underway. Enrichments for iron reducing organisms from the pink layer show precipitation of reduced iron sulfides, indicative of iron or sulfate reduction. Enrichments for manganese reducing organisms show a milky white precipitate (rhodocrocite) indicative of Mn(IV) reduction. In order to characterize the community DNA of the Salt Pond, clone libraries made generated using cyanobacterial and eubacterial specific primers. Libraries were made from DNA extracted from both the pink layer (3.5 meters) and the layer above the pink layer (3.25 meters). A variety of unique RFLP patterns resulted, indicating that both communities are diverse and that there are differences between the communities at the different depths. *Chlorobium phaeovibriodes* sequence was recovered from the pink layer. The existence of such an organism in the pink layer is consistent with other observations including whole cell spectral analysis of pigments and environmental conditions.

Introduction

Salt Pond is a chemically and thermally stratified pond with a unique pink layer at 3.5 meters depth. The pink layer occurs at the oxic-anoxic transition zone (OATZ) and is no more than 0.5 meters thick during stratification. Previous characterization of this site by Bazylinski et al reveals that within the pink layer (OATZ) soluble ferrous iron concentration increases dramatically, and just above the OATZ at 3.25 meters depth, the particulate Fe(III) reaches peak concentrations. Due to the unique distribution of iron in the OATZ, our Microbial Diversity Course group chose to investigate the microbial transformations of iron and manganese within the OATZ using enrichment techniques, and the enrichment portion of this project is a continuation of this group project. Enrichments for iron and manganese reducing bacteria were constructed with the goal of isolating and characterizing organisms that actively contribute to the geochemical cycling of iron and manganese in the pink layer of Salt Pond.

The second portion of this project is concerned with community analysis of DNA from the Salt Pond. To date, researchers have not utilized molecular techniques as a method of characterizing the bacterial community in the Salt Pond. While enrichment culture techniques are useful and informative when researching specific phenotypes or physiotypes, it is often valuable to know what organisms are in a given environment before attempting culture work. Additionally, DNA analysis of a community can offer insights into the diversity of an environment substantially faster than enrichment culture techniques. I chose to characterize the pink layer of the Salt Pond for several reasons. First, preliminary work illustrates that the pink coloration is cell-associated and that pink coloration is correlated with phototrophic metabolism. Additionally, the absorption spectrum of suspended pink layer cells closely resembles that of chlorophyll d, which has only been described in one organism (Miyashita, et al.). The chlorophyll d-containing organism has not been legally described, but is reported in the literature as a cyanobacteria–like or prochloron-like organism.

Four specific questions were addressed in this study: (i) what organisms are present at 3.5 meters depth (the pink layer) and at 3.25 meters depth (just above the pink layer) in the Salt Pond, (ii) qualitatively, which organisms appear to be most dominant at which depth, and how do the communities at different depths differ in diversity, (iii) what organism(s) are candidates for contributing to the pink coloration in the pink layer, and (iv) what organism(s) are likely to be responsible for the unusual pigment spectrum seen in whole cell suspensions? These questions can be addressed by a variety of molecular and microscopic techniques.

Methods and Materials

Site characterization

Salt Pond is a seasonally stratified pond that reaches a maximum depth of 4.5 meters. Limnological depth profiles were obtained for oxygen, salinity, temperature, pH, and manganese at the sampling site (Clorox buoy roughly 300 yards off shore towards osprey nest). Profiles illustrate that oxygen is depleted by 3.5 meters depth, below which the hypolimnion remains anoxic. Salinity increases with depth, with the top layers having a salinity of 18 parts per thousand (ppt) and bottom layers reaching a salinity of 26 ppt. The temperature profile is typical of a seasonally stratified water body, with the thermocline and chemocline occurring concomitantly at 3.5 meters depth. Surface waters have a temperature of 25 degrees Celsius, and bottom waters reach as low as 16°C. pH decreases slightly with depth, ranging form 8.1 at the surface to 6.8 in the hypolimnion. The soluble manganese profile is similar to that of iron as determined by Bazylinski et al. The surface waters show less than 1 uM soluble Mn, with the Mn concentrations peaking at 3.25 meters depth. At 3.25meters, Mn concentrations reach 10 uM, followed by a decrease in concentration to 7uM Mn at the bottom of the pond. Nutrient and metal analysis including nitrate and nitrite, ammonium, phosphate, sulfide, and iron is currently underway.

Enrichment cultures

Enrichment cultures for selection of iron and manganese reducers were made in both freshwater and brackish medium. All enrichment protocols were carried out under strictly anaerobic conditions using Balch tubes and Miller's application of Hungate technique (Miller et al). Freshwater medium included (in g/L): NH_4Cl (0.25), NaCl (1.0), $MgCl_2 \bullet 6H_2O$ (0.4), KCl (0.5), $CaCl_2 \bullet 2H_2O$ (0.15), 1 mL 1000X trace element solution, 20 mM $NaHCO_3$, 1 mL 1000X vitamin solution, 0.01% yeast extract, and 0.1mM cystein-HCl. 4mM $KH_2PO_4$was added to all enrichments except those with $Fe(III)_5 \bullet PO_4$ as the source of Fe(III). 10 mM Na-acetate was used as the electron donor in all enrichments, and all were grown under $15\%H_2$:$75\%CO_2$. Brackish water medium contained the same components, with the only difference being 17.56 g/L NaCl to simulate the ionic strength of salt water.

Three sets of enrichments were constructed using three different electron acceptors. Each set of enrichments consisted of a freshwater and brackish water media. Two different forms of ferric iron were utilized as electron acceptors, amorphous $FeO(OH)_3$ (240mM) and $Fe(III)_5 \bullet PO_4$. The Fe(III) oxyhydroxides were synthesized previously and the method of synthesis is unknown. $Fe(III)_5 \bullet PO_4$ was synthesized using a simple precipitation based on the fact that as Fe(III) compounds precipitate, soluble phosphate is sequestered. Briefly, a 10 mM KH_2PO_4, 50 mM $FeSO_4 \bullet 6H_2O$ solution was neutralized to pH 7 and purged with air at 4°C overnight. A greenish-brown precipitate ($Fe(III)_5 \bullet PO_4$) formed and was washed several times. Precipitate was spun down by centrifugation, but was left as a suspension. Because some of the precipitate was lost in various wash and aspiration steps, the molarity of the suspension was not calculated. 1 mL of the suspension was used for a source of Fe(III). This source of Fe(III) was utilized with the idea that, i the absence of phosphate, only those organisms which could reduced the Fe(III) could gain access to the sequestered phosphate for growth.

Amorphous MnO_2 was used as the source of Mn(IV). This compound was synthesized according to the method of Burdige. Briefly, $KMnO_4$ and $MnCl_2 \bullet 4H_2O$ were combined in alkaline solution over heat, and upon cooling, black precipitate (δMnO_2) formed. The sample was freeze-dried for preservation and 40mM $MnO_2$was used in the media as terminal electron acceptor.

Inoculum was taken from 3.0 meters, 3.5 meters (the pink layer), and 3.75 meters of the Salt Pond and enrichments were incubated at room temperature in the dark.

DNA isolation from water column

DNA was extracted from the Salt Pond water column at two different depths, 3.25 meters, and 3.5 meters (pink layer). Water column samples (300 mL) were centrifuged at 9000x G in order to concentrate cells. The cells were resuspended in 1 mL supernatant and DNA was isolated using the Mo Bio Ultra Clean Soil DNA Isolation Kit or using the Promega Wizard Kit for preparation of genomic DNA. The Mo Bio extraction kit utilizes beadbeating as a method of cell lysis, which can shear the DNA. This kit was found to be most effective if samples were placed in the beadbeater for less than 15 seconds, in order to minimize fragmentation of DNA. The Promega Wizard Kit uses a hot lysis and was found to be very effective in providing clean genomic DNA.

PCR Amplification of DNA

Extracted DNA from 3.25 meters and 3.5 meters was amplified using primers targeted towards 16s rDNA of eubacteria, archea, and cyanobacteria. Primer set sequences were as follows: Eub8F: 5'-AGAGTTTGATCCTGGCTCAG-3' Eub1492R: 5'-GGTTACCTTGTTACGACTT-3' Arc21F: 5'-TTCCGGTTGATCCYGCCGGA-3' Arc915R: 5'-GTGCTCCCCCGCCAATTCCT-3' The cyanobacterial specific primer set was donated by Scott Dawson and the sequence is unknown. The amplification begins at 356F, and the reverse primer used was Eub1492R. PCR from

environmental DNA was amplified using an Eppendorf Mastercycler Gradient PCR Thermocycler. The gradient was set from 45°C to 65°C, with twelve wells spanning these temperatures. Each reaction mixture contained 2.5 uL 10x AmpliTaq Gold Buffer, 2.5 uL 25mM $MgCl_2$, 1.0 uL dNTPs (at 1.25mM concentration of each dNTP), 1 uL (0.1 ug) of both the forward and reverse primer, 0.5 uL (5U/uL) Taq polymerase, 1.25 uL 1%BSA in water (to bind humics), and 1 uL template (10-100 ng DNA). The remainder of the reaction was filled to 25 uL total volume with sterile, UV cross-linked ddH_2O. PCR cycles were programmed as follows: 95°C for 12 min, [95°C for 30 sec, annealing temperature (45-65°C) 30 seconds, 72°C for 45 sec] x 30 cycles, 72°C for 3 minutes, 4°C hold. Amplified product was visualized on 1.25% SeaKem agarose gels (made in 0.5x TBE buffer), using GelStar stain as the intercalating agent to visualize bands of DNA under UV light. Gel electrophoresis was run at 80-100V.

Creation of clone library from PCR products

PCR products were cloned into *E. coli* using the Invitrogen TOPO TA Cloning Kit. Fresh PCR products were ligated into pCR2.1 vector in a five minute ligation as described in kit instructions. One Shot competent cells were used for transformation into *E. coli*, and kit instructions were followed for transformation. Transformants were plated on LB/amp plates overlain with 40 uL of 40mg/mL X-gal. Plates were incubated at 37°C overnight and observed for the presence of white colonies. Clone libraries were stored at 4°C for future use.

Screening clone libraries for unique clones

The 16s rDNA fragments in the transformed vector were amplified from whole colonies using primers specific to the vector, pRC2.1. Whole colony PCR was preformed by touching a pipette tip to a white colony from the library and dipping the pipette tip into a microcentrifuge tube

containing 25 uL of PCR reaction mix. Reaction mix consisted of the same reagents as previously described with two exceptions. First, the forward and reverse primers were TopoF and TopoR (specific for the vector) or M13F and M13R (also specific for the vector) instead of primers specific to a group of organisms. Secondly, the volume of template was considered negligible (as it was in colony form) and therefore 15.55 uL of sterile ddH_2O was added to bring the volume up to 25 uL total. After PCR, products were visualized as before.

10 uL of PCR product were utilized for restriction fragment length polymorphism (RFLP) analysis to determine unique clones. Restriction digest mixtures contained 10 uL PCR product, 2 uL NEB2 10x buffer, 0.2 uL HinP1 (10U/uL), 0.1 uL Msp1 (20U/uL), and 8 uL sterile ddH_2O. PCR cycles were the same as those run before, with the annealing temperature fixed at 50°C or 57°C. Digestion reactions were incubated at 37°C for a minimum of 3 hours (some digests went overnight) and RFLP patterns were visualized on 1.5% Metaphor agarose gels using GelStar as the DNA stain. Gel electrophoresis was run at 70V.

Sequencing

PCR products showing unique RFLP patterns were sent to Bruce Paster at the Forsythe Dental Institute for sequencing. PCR products were also sequenced locally by Scott Dawson and Joel Klappenbach using an ABI 310 Automated Sequencer. Sequencing was attempted using both raw PCR product and well as PCR product run through a Centricon spin column. A variety of sequencing primers were utilized depending on the original primer used for amplification. Sequences were returned as text files.

Creating a phylogenetic tree

Sequences were first taken through a sequence match using either the BLAST search (http://www.ncbi.nlm.nih.gov/Recipon/index.html) or the Ribosomal Database Project (RDP) (http://www.cme.msu.edu/RDP/) to find sequences which may prove useful for manual alignment. The ARB program developed at the Technische Universitat Munchen (http://www.biol.chemie.tu-muenchen.de) was the phylogenetic packaged used to align sequences and create phylogenetic trees. The ends of sequences were removed and the Fast Aligner V1.02 in ARB_EDIT4 (the new ARB editor) was used to initially align sequences. Sequences were aligned using the pt_server SSU_rRNA.arb. After initial alignment, sequences were placed within the phylogenetic tree using the "add species to existing tree" option, followed by the "quick add marked to a tree using parsimony". Bacterial filters were used during alignment for all cloned sequences originally amplified with eubacterial or cyanobacterial specific primers. Often the ends of the sequences were not aligned correctly and thus the resulting placement in the phylogenetic tree appeared suspect (long branch length for example). Sequences were then manually aligned using sequences suggested by BLAST, ARB and by the original alignment. Once properly aligned, the sequence data was used to generate phylogenetic trees. The neighbor joining method was utilized to create trees from a specific set of marked sequences. Again, the bacterial filter was used in tree creation.

Alignment of secondary structure

Once the sequence had been placed in the phylogenetic tree, the sequence was manually aligned to the secondary structure of a close relative. This alignment is intended to illustrate compensatory base pair changes as well as differences in stem length and loop structure. Alignment is begun in a conserved region (generally a well-conserved loop) and proceeds forwards and backwards along the sequence.

Results and Discussion

Iron and Manganese Reducing Enrichments

After one week, iron-reducing cultures showed an increase in the amount of black precipitate present (presumably reduced iron sulfides), with a corresponding decrease in the amount of insoluble Fe(III) present (as determined by visual observation). Manganese reducing enrichments showed no visible signs of solubilization of MnO_2 after only one week. However, after several weeks, a milky white precipitate (presumably rhodocrocite, reduced manganese carbonate) formed. In both iron and manganese reducing enrichments, microscopic inspection revealed actively motile cells. In all cases, color changes within the cultures appeared most rapidly in the 3.5 m samples, followed by the 3.0 m samples and lastly by the 4.0 m samples. Enrichments were transferred and further characterization has not yet taken place.

Isolation of DNA and PCR amplification

DNA was successfully isolated from the Salt Pond water column (3.24 m and 3.5 m) using either the Mo Bio or Promega DNA extraction kits. Gradient PCR over temperatures ranging from 45°C to 65°C gave single clear bands at the correct weight for both eubacterial and cyanobacterial primers (see Fig.1). However, amplification in this temperature range did not produce a single band when archeal primers were used (see Fig, 1).

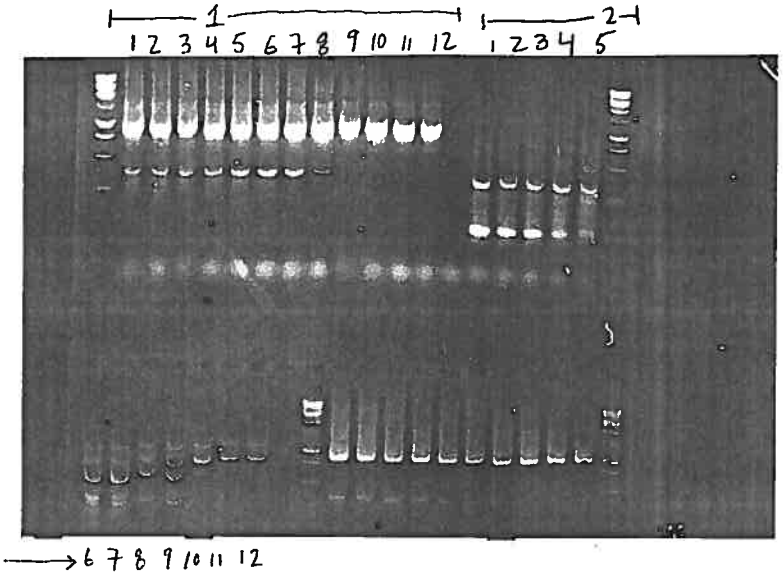

Fig 1. PCR amplification of DNA from 3.5 m of Salt Pond using cyano356F-Eub1492R (1) and using Arc21F-Arc915R (2). Well 1 is amplification at 45°C with the gradient extending to well 12 at 65°C. Amplification is specific for cyanobacterial primers but not for archeal primers.

Archeal primers may require a higher temperature range in order to amplify specifically. At this point in the project, the unique pigment spectrum of whole cell suspensions (Eric Giados and Brent Christner, personal communication) was discovered, and a literature search revealed that organisms with similar spectra containing either bacteriochlorophyll e or chlorophyll d were likely to be either eubacterial or cyanobacteria-like. For this reason, further experiments were focused on investigation of eubacteria and cyanobacteria present in the Salt Pond water column.

Ligation and transformation

Fresh PCR products from eubacterial and cyanobacterial primers at 3.25 and 3.5 m depth were ligated into the pCR21 vector provided in the TOPO TA cloning kit and ligation products were transformed into OneShot competent cells. After growth overnight, the plates containing eubacterial clones showed roughly 25 white colonies. The plates containing cyanobacterial clones from each depth, however, showed over 100 white colonies per plate. Because the ligation and

transformation efficiency of the cyanobacterial PCR product, work primarily focused on these cones. However, some work was continued with eubacterial clones as well.

PCR amplification of insert from the TOPO vector

Individual clones were picked for RFLP analysis. However, before RFLP can be utilized, the insert must be amplified by primers specific for the vector which will amplify through the insert. This allows the digestion substrate to be amplified so that more digestion product will be visible. Additionally, it allows you to be certain that the clones have insert and that the insert is the correct size. Eight clones were chosen from the cyanobacterial library (3.5 m depth) and amplified using TopoF and TopoR primers. The results of this amplification are shown in Figure 2.

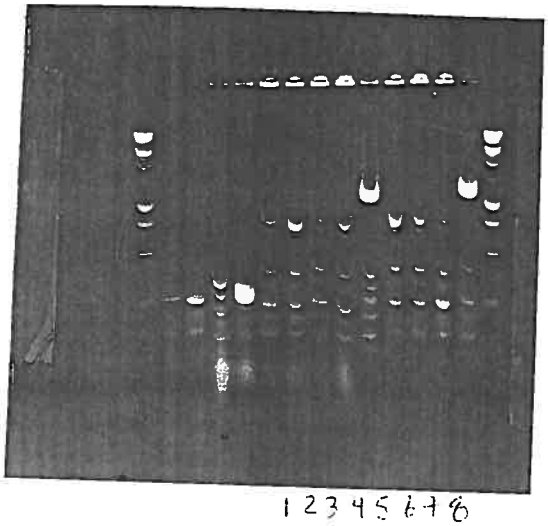

Fig. 2. 8 clones from cyanobacterial library (3.5 m) amplified with TopoF and TopoR primers.

It is clear that the amplification did not produce the expected single band of 1 kb. Each colony harbors only one plasmid, and each plasmid should contain only one inserted sequence. Even is the insert was the incorrect size, amplification should only produce one single band. Several plausible explanations were investigated. First, the annealing temperature was raised from

50°C to 57°C. This did not increase the quality of the PCR product. Next, the PCR reagents were exchanged for new reagents with the reasoning that possibly one or more reagent was contaminated with vector. This also produced a similar result. Lastly, new primers (M13F and M13R) specific to another portion of the vector were utilized. As shown in Figure 3, these primers produced very clean bands from individual colonies.

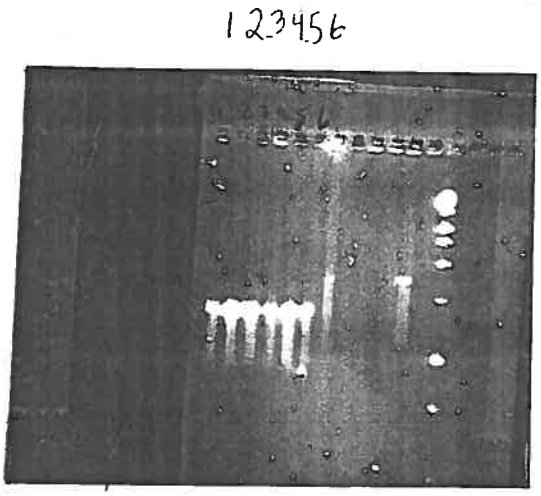

Figure 3. 6 clones from cyanobacterial library (3.5 m) amplified with M13F and M13R primers.

RFLP analysis

The PCR products of the 6 clones shown in Fig. 3 were digested using HinP1 and Msp1. The resulting RFLP patterns are shown in Figure 4.

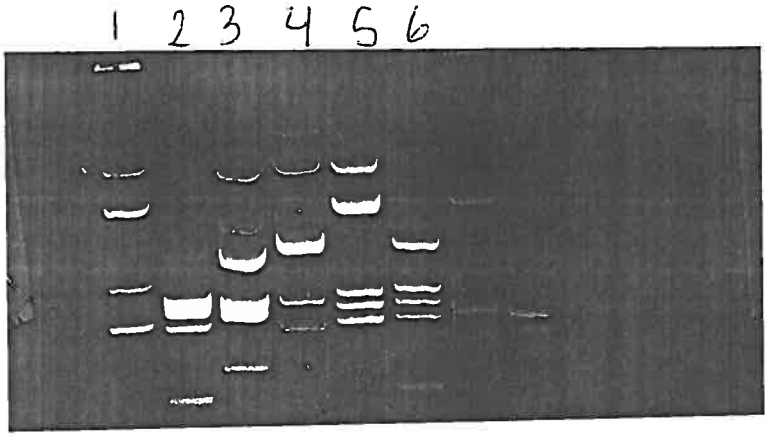

Figure 4. RFLP analysis of 6 clones shown in Fig. 3.

The RFLP analysis illustrates 6 unique patterns, which suggests 6 different types of sequences. This shows that not only is the community fairly diverse, but that many more clones must be screened before we have exhausted the diversity of sequences found in the pink layer of Salt Pond. In addition to this RFLP analysis, RFLP was preformed on eubacterial plates as well as cyanobacterial plates from 3.25 meters depth. The eubacterial clones (3.5 m) showed 4 different RFLP patterns, with two of the 4 patterns occurring at a much greater frequency than the others did. Four cyanobacterial (3.25 m) clones were analyzed and all showed unique RFLP patterns in comparison to each other as well an in comparison to the pink layer RFLP patterns.

Sequencing

In total, 6 cyanobacterial clones from 3.5 m depth (see Fig. 3 and 4), 4 cyanobacterial clones from 3.25 m depth. and 4 eubacterial clones from 3.5 m depth were sent for sequencing. Many clones were sent in duplicate. Only one sequence (eubacterial – 3.5 m depth) was successfully obtained. This clone was sent in duplicate to the Forsythe Dental Institute and was given to Scott Dawson in

duplicate for local sequencing. Thus, 4 different sequence reads (Allison0 - Allison3)from the same clone were retrieved.

Creating a phylogenetic tree

Sequence data was imported into ARB and aligned as detailed in the description of the methods. The resulting tree presented in Fig. 5 shows that this clone falls into the *Chlorobium* group, with its closest relative being *Chlorobium phaeovibriodes*. Additionally, all 4 sequences from the same clone produce very similar results, indicating that the sequences were clean.

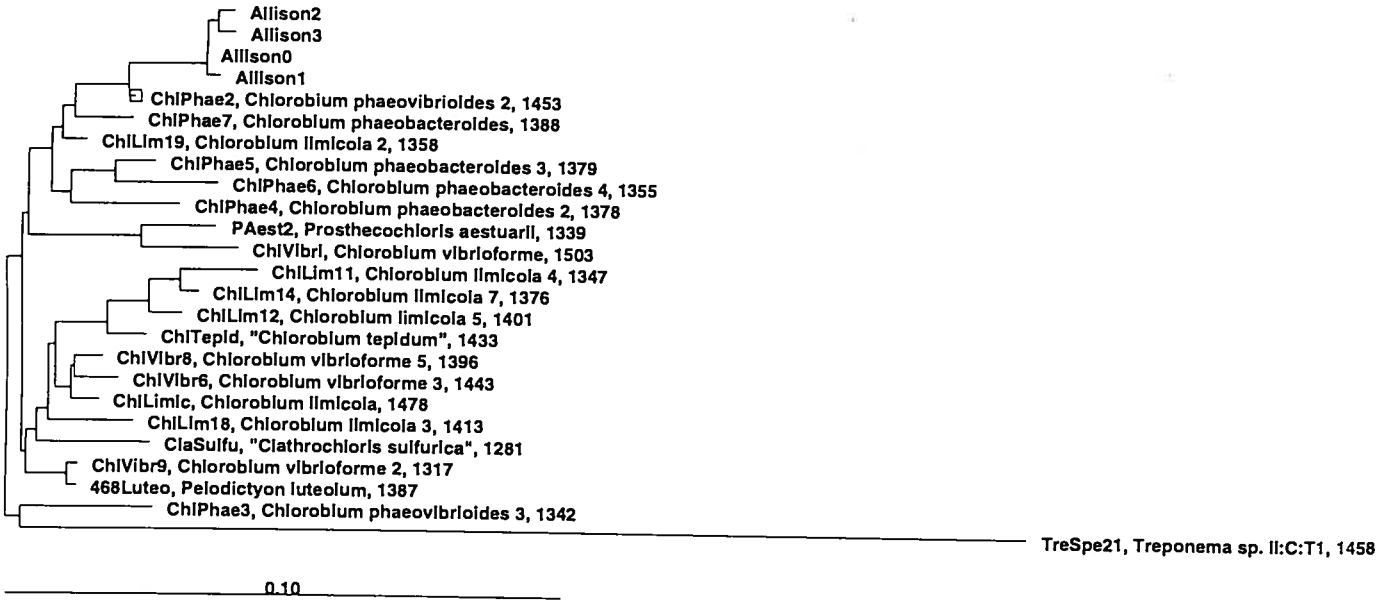

Fig. 5. A phylogenetic tree including a Salt Pond clone from the pink layer (3.5m), Allison

Alignment of secondary structure

Manual alignment of the secondary structure of rRNA from Allison0 sequence to that of *Chlorobium vibrioforme* is presented in Fig. 6. Manual alignment reveals compensatory base pair changes that serve to preserve secondary structure. All bases that differ from the sequence of Chlorobium vibrioforme are shown.

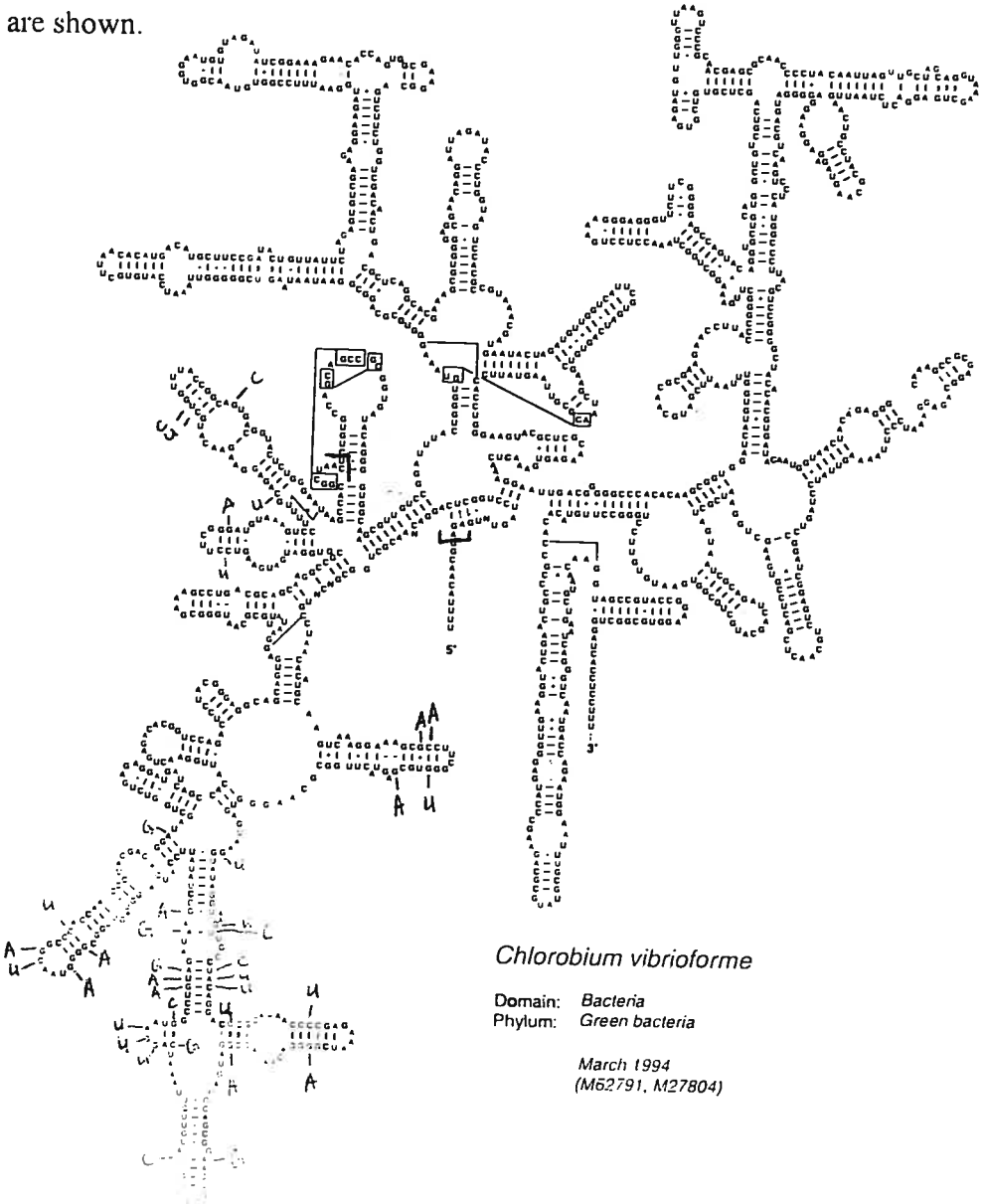

Fig 6. Manual alignment of 16s rDNA sequence of Allison0 to that of *Chlorobium vibrioforme* showing unique nucleotides.

Conclusions

The existence of *Chlorobium phaeovibriodes* in the pink layer of Salt Pond is reasonable for several reasons. First, members of the green sulfur bacteria, such as *Chlorobium*, carry out anoxygenic photosynthesis using H_2S as their source of electrons. Limnological profiles indicate that by 3.5m depth, oxygen is depleted. Additionally, the sulfide concentration is elevated under the pink layer, but the sulfide is depleted within the pink layer (see data from Dollhoff, Sherry). This pattern is consistent with the physiological needs of *Chlorobium* species. Examination of the whole cell spectrum of a pure culture of *Chlorobium phaeobacteriodes* reveals that a peak is present at 723 nm and the spectrum of *Chlorobium phaeobacteriodes* is similar to that of whole cells suspensions of the pink layer. *Chlorobium phaeovibriodes* harbors bacteriochlorophyll e as its major bacteriochlorophyll, and bacteriocholorophyll e is the pigment responsible for the unique peak at 723 nm in the cell suspension of *Chlorobium phaeobacteriodes*.

An additional experiment also provides supporting evidence for *Chlorobium phaeovibriodes* contributing to the pink layer. Inoculum from the pink layer was placed in two large identical Winogradsky-type columns. One column was incubated in the dark, while the other was incubated under a 14hour/10hour light/dark cycle. Although the inoculum was identical and was pink at the time of inoculation, after three weeks incubation the column grown in the dark had lost all pink coloration. The column incubated in the light, however, retained the dark pink/red pigmentation associated with the pink layer. This is evidence that the organism(s) contributing to the unique coloration are light dependent. This observation supports the idea that photosynthetic organisms are present in the pink layer and that the pink coloration is associated with photosynthesis. All observed evidence suggests that a bacteriochlorophyll e containing photosynthetic organism capable of utilizing H_2S such as *Chlorobium phaeovibriodes* exists in and may actively contribute to the coloration of the pink layer.

References

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