Enrichment for dissimulatory nitrate reducing and manganese oxidizing bacteria from Salt Pond, Falmouth, MA

Caroline B. Tuit
Microbial Diversity
Marine Biological Laboratory
July 29, 1999

Abstract

Enrichment culture techniques were applied to isolate dissimilatory nitrate reducing and Mn oxidizing bacteria from the oxic/anoxic transition zone of Salt Pond. Nitrate reducers were enriched, were not isolated. Mn oxidation was not shown to occur in the enrichments, but aerobic succinate utilization supported enrichment of a non-phenotypic spirochete species.

Introduction

Salt Pond, located between Mill and Elm roads in Falmouth, MA, is a brackish, redox stratified lake with partial access to the ocean. The Salt Pond water column profiles have been measured for dissolved salinity, pH, temperature, dissolved oxygen, hydrogen sulfide, nitrate+nitrite, phosphate and ammonia concentrations (Figure 1). Salinity varies from approximately half that of seawater in the upper portion of the lake the to two-thirds seawater salinity at depth. This density contrast makes the stratification in the lake extremely stable. The bottom waters of the pond are anoxic with H_2S concentrations up to 4.7 mM. Oxygen concentrations in surface waters are in atmospheric equilibrium but decrease with depth. The sulfide and oxygen profiles overlap at a depth of about 3.5 m. At this redox transition zone, there is a large population of sulfur utilizing phototrophs, forming a turbid pink layer.

The redox stratification of this coastal pond makes it an ideal location to study processes, such as denitrification and manganese (Mn) oxidation that require low ambient oxygen concentrations. These processes occur in many redox stratified environments, such as marine sediments and microbial mats, but

the lower organic matter content of the lake has expanded the suboxic transition zone from the micron to decimeter scale, allowing us to separate these processes. While denitrification has long been recognized as an important anaerobic pathway for carbon degradation, the manganese oxidation pathway has only started to be investigated in the past three decades. The geochemical importance of this pathway is still unknown.

This project attempted to enrich for nitrate reducing and manganese oxidizing bacteria. The dissolved Mn profile was measured in the lake to further characterize the chemical environment. Inoculum for enrichment cultures were collected from both within the pink layer (3.5 m) and from the low oxygen, sulfide-free waters directly above (3.25 m).

Nitrate Reducing Enrichments

Background: Nitrate reduction is the process of converting nitrate to nitrite. There are two types of nitrate reduction, assimilatory nitrate reduction which is performed by any organism using nitrate as it's nitrogen source regardless of the oxidant it is utilizing for energy and dissimilatory nitrate reduction which is performed by organisms using nitrate as an electron acceptor. Dissimulatory nitrate reduction is the first step in the denitrification pathway,

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Denitrification is an anaerobic respiratory process coupled with the generation of ATP. Because nitrite formation could be a product of either assimilatory or dissimilatory nitrate reduction, the formal definition of denitrification requires

reduction of nitrate all the way to the gaseous products N_2O and N_2. Each step in the denitrification pathway is enzymatically catalyzed and depending on the environmental conditions (Eh, pH, organic matter concentration, O_2) the reaction can be terminated at any one of these steps.

Discrepancies in the kinetics of the reactions can also lead to the build up of intermediate products. For example, in *Pseduomonas nautica* an extremely common marine denitrifier, the relative rates of nitrate and nitrite reduction result in the accumulation of nitrite in the media (Bonin, et al., 1987.) This project focussed on isolating organisms capable of dissimulatory nitrate reduction. The tests for dissimulatory nitrate reductase activity employed in this study included both gas formation and nitrite accumulation.

Methods: Denitrifying bacteria and thus dissimulatory nitrate reducers were enriched for in a 40 ml liquid culture screw capped tube. Each tube was inoculated with 0.4 ml of cells concentrated from 300 ml via centrifugation and resuspended in 20 ml sterile half-strength seawater. Inoculum was collected from 3.25 m depth in the suboxic zone and from 3.5 m depth, which is within the anoxic pink layer. Four replicate enrichment cultures were inoculated from each depth. The enrichment media was a two-thirds seawater base with added $NaNO_3$ (50 mM), K_2PO_4 (5.0 mM) and 1 ml/L of the standard vitamin solution. The standard trace element solution was modified to yield a final $MoO_4^=$ concentration of 0.6 µM, approximately 6 times normal sea water concentration. This was included to ensure that nitrate reductase enzyme production, which contains a Mo atom at the site of active oxygen transfer, would not be limited by metal availability. The original enrichment was made using a mixed carbon

source of 20 mM sodium acetate and 20 mM Na succinate. These were chosen as relatively non-fermentable substrates.

Denitrification activity in the original enrichment cultures was monitored by gas formation. Each enrichment culture included and upside down Durham tube to catch any gas produced by the culture. After about a weak of incubation at ambient temperatures and pressures, in the presence of light all cultures showed increased turbidity and slight bubble formation, but when examined under the microscope were found to be infested by ciliated eukaryotes (Figure 2). To prevent the ciliates from grazing out the denitrifiers 50 mg/15 ml of cyclohexamide were added to the cultures. HPLC measurements at that time indicated that acetate and succinate concentrations had been depleted, therefore an additional 40 mM of acetate was added to each culture. These cultures were allowed to incubate for another five days and continued to produce gas. When checked again under the microscope, they exhibited a dense mixed culture of small rods and vibrios bacteria (Figure 3). In order to isolate the denitrifying organisms, dilution to extinction series were inoculated from five of the enrichment cultures. These cultures were incubated under anaerobic conditions in crimped and stoppered vials. The media was prepared using anaerobic techniques. The dilution series were incubated at 28 °C in the dark for 4 days. Very little gas formation occurred. After 3 days, 50 ppm of yeast extract was added to each tube in an attempt to stimulate more denitrifying activity. This resulted in increased bubble formation and turbidity. After 7 days each culture was examined for bubble formation, gas pressure and nitrite accumulation as measured by formation of a blood red complex when reacted with sulfanilic acid and α-naphthlyamine.

Results: Results from the dilution series tests are presented in Table 1. Though many of the tubes showed a positive response to the nitrite test, this response did not follow any pattern. When tested many of the tubes that did not show nitrate accumulation exhibited pH values of less than 6.0 and down to pH 1. This drop in pH has probably poisoned the culture. This was surprising since this system was both buffered with 5 mM K_2HPO_4 and neither the assimilatory and dissimilatory reactions are expected to produce H^+ ions.

Assimilation: $17CH_3COO^- + 15H^+ \rightarrow 8C_4H_7O_3 + 4H_2O + 2HCO_3^-$

Dissimilation: $4NO_3^- + CH_3COO^- \rightarrow 4NO_2^- + 2HCO_3^- + H^+$

$2NO_3^- + CH_3COO^- + 31H^+ \rightarrow N_2O(g) + 2HCO_3^-$

$8NO_3^- + 5CH_3COO^- + 3H^+ \rightarrow 4N_2(g) + 10HCO_3^- + 4H_2O$

The decrease in pH also suggests that the bubbles collected by the Durham tubes may be carbon dioxide rather than a nitrogen species. The dilution experiment needs to be repeated in a media with a higher buffering capacity, in order to isolate dissimilatory nitrate reducing bacteria. However, when the high dilution samples with nitrite activity are examined under the microscope the predominant phenotype is a short, motile curved rod. Organisms capable of using nitrate for denitrification that exhibit such phenotypes include both *Pseudomonas*, and *Vibrio*, both are common in the marine environment.

Manganese Oxidizing Enrichments.

Background: The abiotic oxidation of Mn^{2+} to $MnO_2(s)$ is thermodynamically favored in the presence of oxygen, but kinetically slow at ambient Mn^{2+} concentrations and neutral pH. Microbes have been found to catalyze this process at the suboxic interface in stratified water columns (Tebo et al., 1984). Salt pond represents the perfect environment to study this process and to attempt to isolate manganese oxidizing organisms.

Methods: The water column profile for Mn^{2+} was measured via Atomic Absorption spectroscopy (Figure 4). Concentrations were calculated using a synthetic seawater standard curve to correct for matrix effects. Water samples were filtered to remove particulate material and acidified to pH 2 for storage. Procedural blanks were below detection limits. Mn^{2+} concentrations range from 0.24 µM in the oxic zone to 10 µM at 3.25 m and decreases to about 7.6 µM in the anoxic portion, probably due to scavenging by sulfide minerals or organic matter. This profile is typical of those seen for Mn^{2+} in stratified water columns. The highest rate of Mn^{2+} oxidation should occur directly above to oxic/anoxic transition, from about 3.0 to 3.25 m.

Samples used to inoculate Mn oxidizing enrichments were taken from 3.25 and 3.5 m. The enrichment media used was the Mn Minimal Media used by Kepkay and Nealson (1989) for continuous culture of the manganese oxidizer *Pseudomonas putida*, one of the few papers were Mn oxidation appears to be linked with carbon utilization. This media is a two-thirds seawater base with additions of 9.0 mM NH_4Cl, 0.4 mM $NaHCO_3$, 2.0 mM K_2HPO_4, 10.0 mM Hepes buffer, 1 ml standard vitamin solution, 5 µM $Fe(II)SO_4 \bullet 7H_2O$ and 5 to 10 mM Na succinate as the carbon source. Manganese concentration is varied from 5µM to

10 mM. This is a well buffered media maintained a pH of 7.8, well below the pH required for autocatalytic Mn oxide formation.

Both liquid and gradient enrichments were established. The initial gradient enrichment was designed with 10 μM Mn^{2+} in a 1.5% agar plug in the bottom of the tube. This was topped with a 0.6% agar media containing succinate, but no Mn^{2+} and inoculated with 100 μL. Tubes were stoppered to reduce oxygen content but no precautions were taken to avoid oxygen during preparation. Cultures were incubated in the dark at 28°C. All tubes exhibited growth with two days of inoculation. Colony morphology ranged from diffuse yellow colonies to small white ones. After five days, the colonies were picked for molecular analysis. Microscopic observation revealed that none of the colonies were pure cultures (Figure 5). To obtain sequence information, DNA was amplified using direct colony polymerase chain reaction (PCR) of two colonies, a diffuse light orange colony and a small white colony both from 3.25 m. The DNA was amplified using eubacteriral 8F 1492R primers and cloned using the invitrogen TOPO cloning kit. 12 clones were picked from each sample for restriction fragment length polymorphism RFLP PCR. PCR product was obtained for 11 out of 12 clones from the diffuse colony but not for any clones from the small white colony. RFLP analysis of the diffuse colony clone library yielded and 4 distinct patterns (Figure 6). One of the samples was sequenced and when treed using the ARB database aligned within the *Spirochaete* (Figure 7).

All of the picked colonies were transferred to two new sets of gradient tubes. The first contained 10 mM of Mn^{2+} in the plug, with succinate in the overlying media colonies with morphologies similar to those transferred grew

with 2 days of inoculation. The second gradient tubes the bottom plug contained both 10 mM Mn^{2+} and 5 mM succinate. This ensured that the energy and carbon source diffused together. All of these tubes had growth within two days. None of the gradient enrichments should precipitated any Mn oxides.

Liquid cultures containing 5 μM and 50μM Mn were inoculated from 100 μL of Salt Pond cell suspensions. After two weeks all tubes showed growth with greater turbidity in the 5 μM cultures. Some tubes formed small quantities of a black precipitate, however AA analysis of Mn concentrations showed that all cultures were with 10% of the Mn concentrations in the abiotic controls. The black precipitate is most probably iron sulfide.

Results: Neither gradient tubes or liquid enrichments exhibited any indication that manganese oxidation was occurring in these cultures. A comparison of the relative energy yield of Mn oxidation versus succinate oxidation more favorable per mole of oxygen shows that succinate oxidation is almost 3 times more favorable. This suggests that what was isolate was a succinate utilizing aerobe rather than a Mn oxidizer.

The isolated sequence was most similar to three uncultivated eubacteria clones isolated from the methanogenic region of a petroleum contaminated aquifer by Dojka et al., (1998.) Microscopic examination of this colony showed many short curved rods, but had not revealed any organisms with spirochete morphology (Figure 5). At this time, *Spirochaete* is the only phylogenetic group considered to have a consistent morphology. These results suggest that either microscope analysis missed the spirochetes present in this sample or that this is another case of discrepancy between phenotypic and phylogenetic classification.

Other labs have recently identified phylogenetic spirochetes that do not exhibit spirochete morphology (Sherry Dollhopf, pers com.)

Conclusions

This project succeeded in enriching for organisms capable of dissimilatory nitrate reduction but did not achieve an isolate. These organisms were small curved rods and are mostly likely members of the *Vibrios* or *Pseudomonas* groups, both of which are common in the marine environment. Enrichments for Mn oxidizing bacteria were not successful. Growth on succinate with alternative electron acceptors (O_2, or $SO_4^=$) appears to have predominated. Isolates sequenced revealed a non-phenotypic *Spirochaete.*

References

Kepkay, P. E. and Nealson, K. H. (1989) Growth of a manganese oxidizing Pseudomonas sp. in continuous culture.*Arch. Microbiol.* **148**, 63-67,.

Bonin, P., Gilewicz, M. and Bertrand, J. C. (1987) Denitrification by a marine bacterium *Pseudomonas Nautica* strain 617. *Ann. Inst. Pasteur/Microbiol.* **138**, 371-383.

Tebo, B. M, Nealson, K. H., Emerson, S. and Jacobs, L. (1984) Microbial mediation of Mn(II) and Co(II) precipitation at the O_2/H_2S interfaces in two anoxic fjords. *Limnol. Oceanogr.* **29**, 1247-1258.

Figure Captions

Figure 1. Water column Profiles of chemical properties of Salt Pond. Nitrate+nitrite, ammonia and phosphate were measured by Yvonne Van Lith, dissolve oxygen salinity and temperature were measured by Group III (Brent Christner, Christopher Chyba, Allison Niggemyer, and Yvone Van Lith) and sulfide concentrations were measured by Sherry Dollhpf.

Figure 2. Ciliate found in 3.5 m salt pond enrichment.

Figure 3. Mixture of small rods and vibrios forms from 3.5 m salt pond enrichment after treatment with cyclohexamide.

Figure 4. Dissolved Mn^{2+} water column profile and standard curve from AA analysis.

Figure 5. Diffuse light orange colony picked from 3.25 m Mn oxidizing enrichment culture.

Figure 6. RFLP analysis of the diffuse light orange colony, yielding 4 distinct pattern. + indicates patterns submitted for sequencing. Arrow indicates sequence obtained.

Figure 7. Phylogenetic tree obtained from the ARB database for the RFLP pattern shown in Fig. 6. The sequence falls within the *Spirochaete* group.

Figure 1.

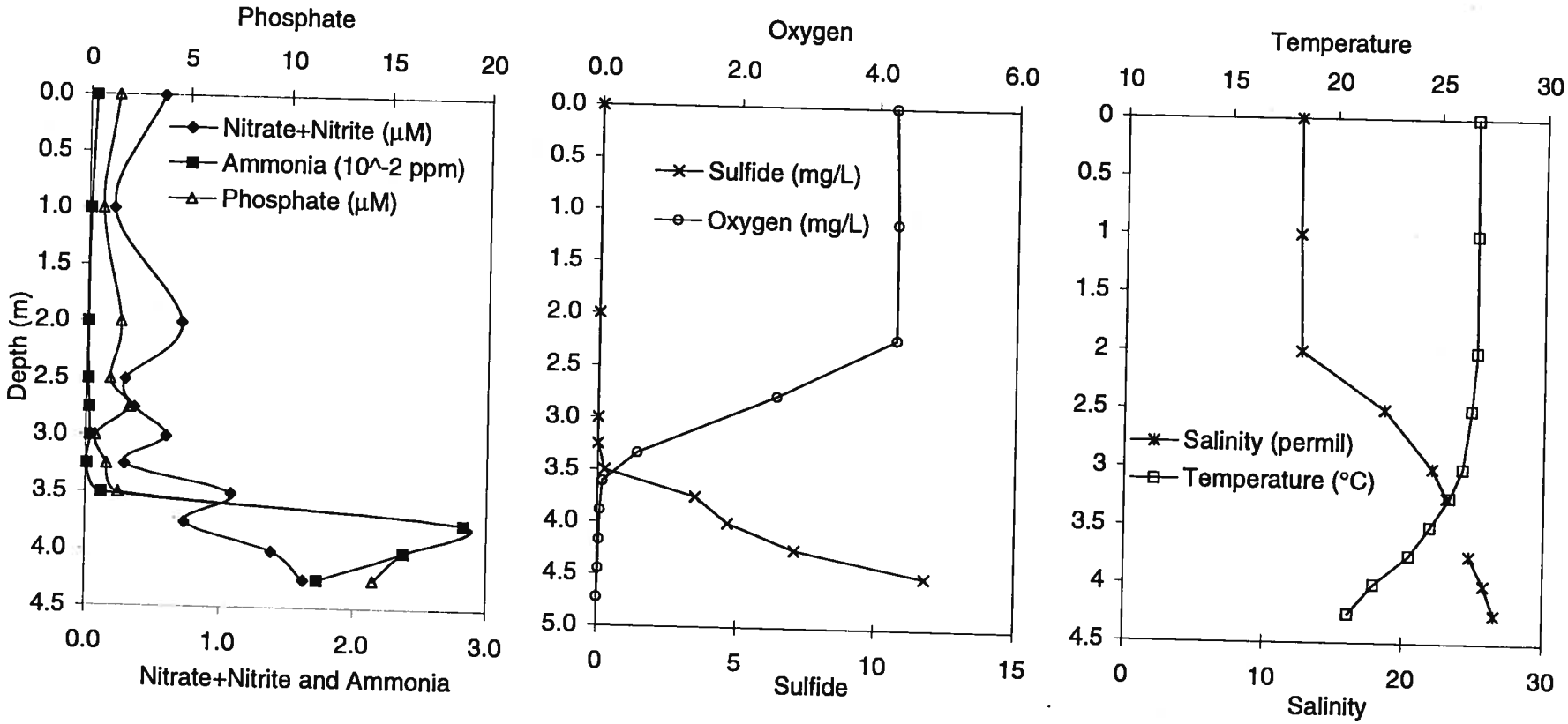

Phosphate
Nitrate+Nitrite (µM)
Ammonia (10^-2 ppm)
Phosphate (µM)
Depth (m)
Nitrate+Nitrite and Ammonia
Oxygen
Sulfide (mg/L)
Oxygen (mg/L)
Sulfide
Temperature
Salinity (permil)
Temperature (°C)
Salinity

Figure 2.

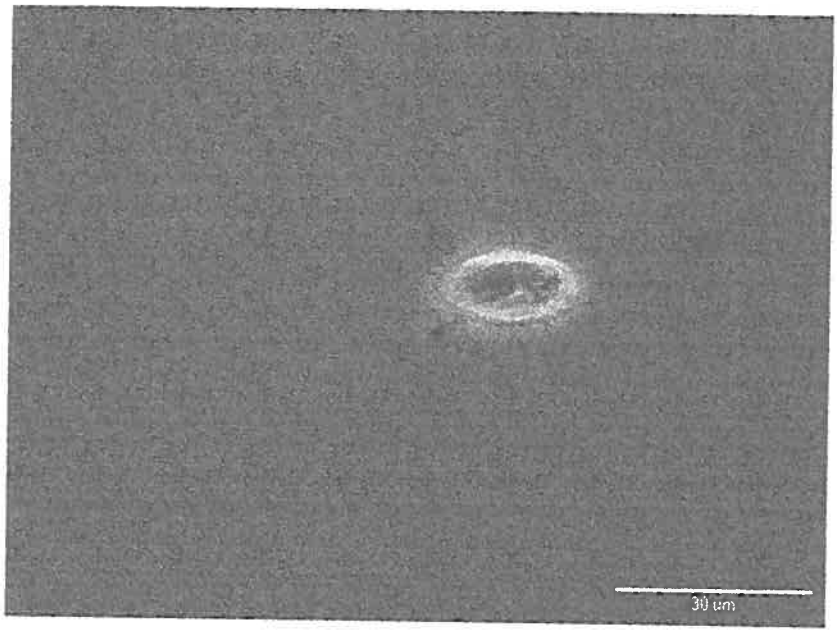


Figure 3.

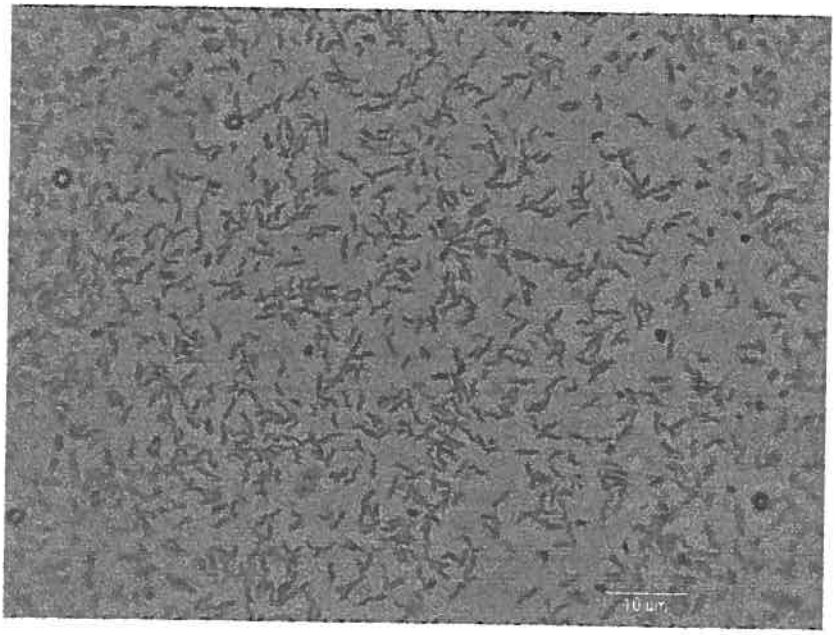


Figure 4.

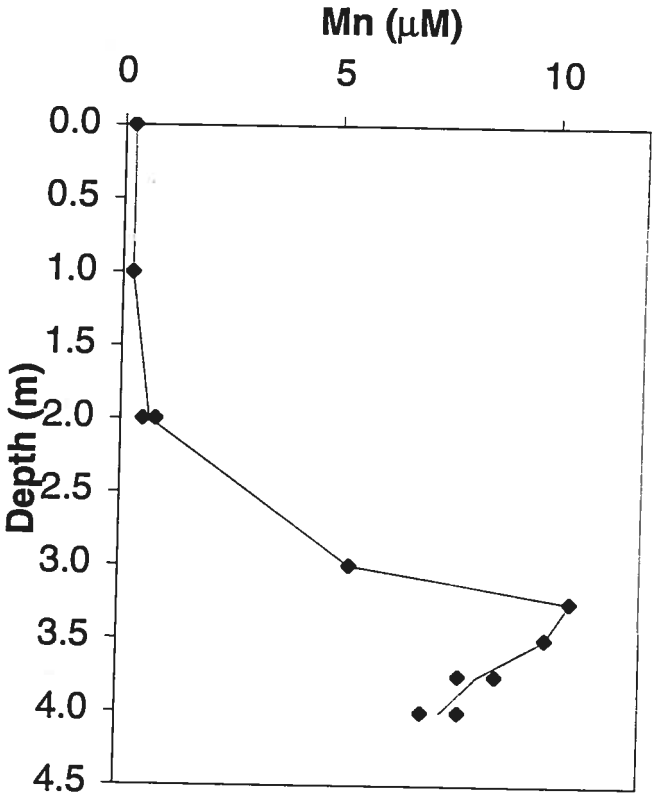


Figure 5.

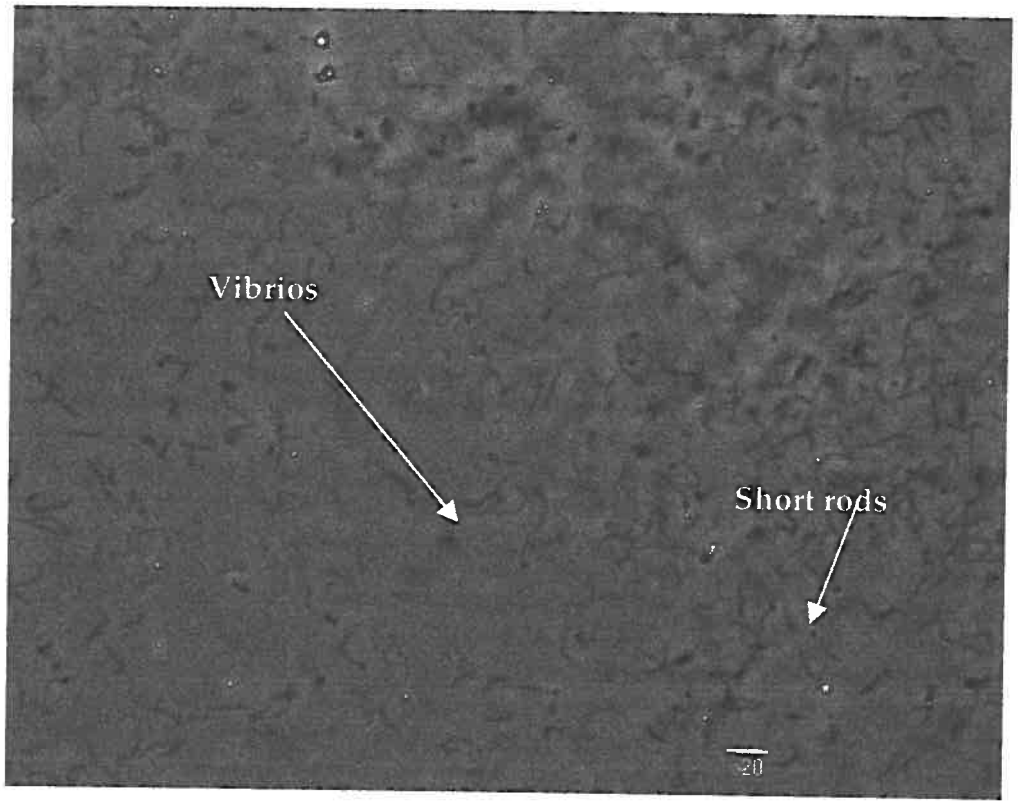
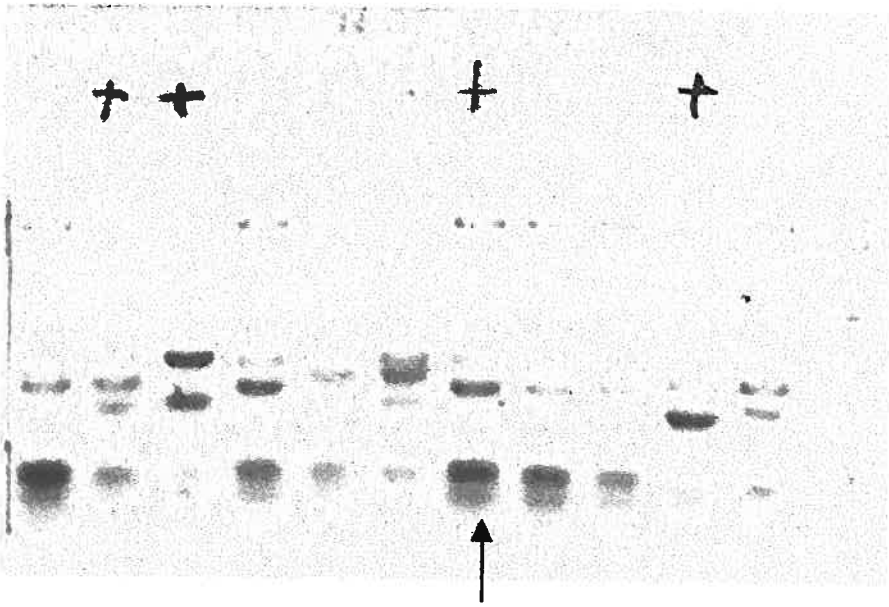
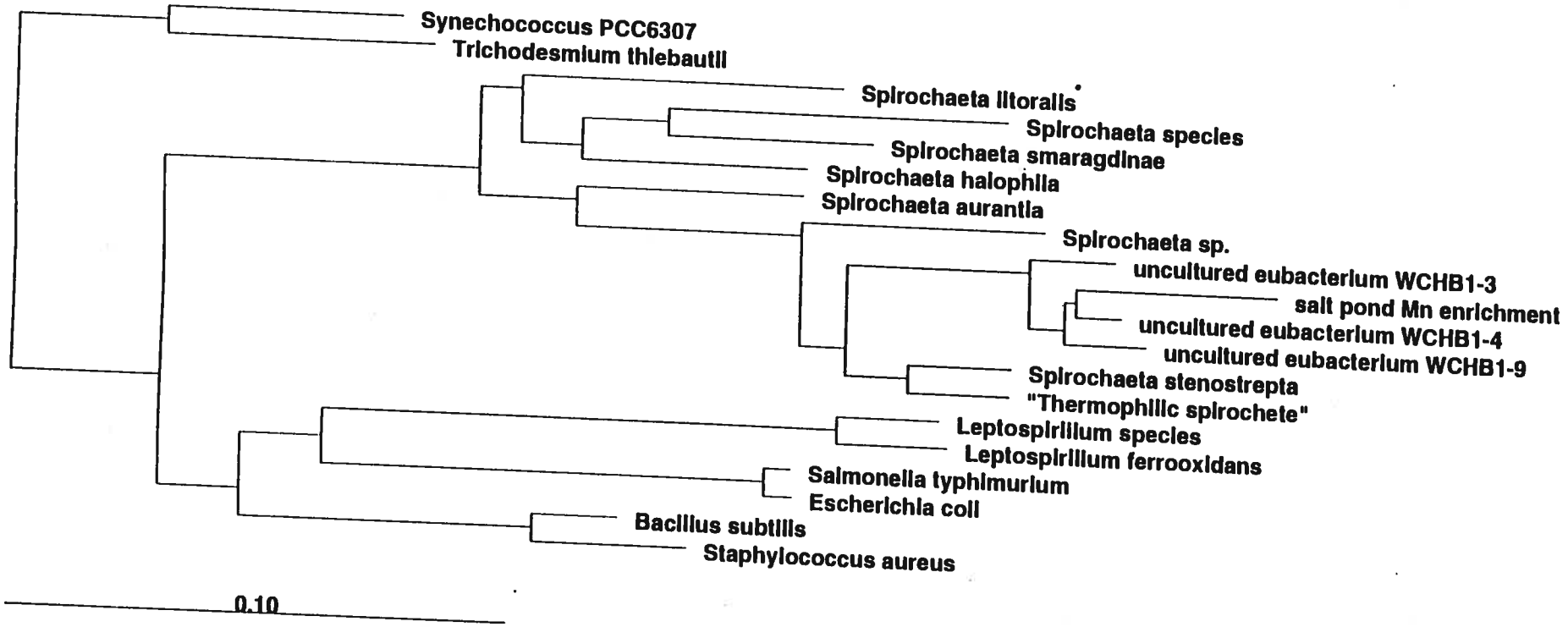


Figure 6.



Spirochaeta Sequence


Synechococcus PCC6307
Trichodesmium thiebautii
Spirochaeta litoralis
Spirochaeta species
Spirochaeta smaragdinae
Spirochaeta halophila
Spirochaeta aurantia
Spirochaeta sp.
uncultured eubacterium WCHB1-3
salt pond Mn enrichment
uncultured eubacterium WCHB1-4
uncultured eubacterium WCHB1-9
Spirochaeta stenostrepta
"Thermophilic spirochete"
Leptospirillum species
Leptospirillum ferrooxidans
Salmonella typhimurium
Escherichia coli
Bacillus subtilis
Staphylococcus aureus
0.10