

Microbial Diversity Course 1998
Woods Hole, MA

Characterization of microbial populations degrading aromatic monomers in aerobic and anaerobic enrichments with Salt Pond sediments, a preliminary study

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Abstract

Aerobic enrichments with sediments from Salt Pond, MA, with carbon and energy sources provided as syringic acid, salicylic acid, vanillic acid and benzoic acid, at a concentration of 1 mM each, showed a complete degradation of these substrate after 3 days of incubation at 30°C. In the control with the media without sediments, no degradation occurs. In anaerobic conditions the degradation started after 7 days of incubation. MPN gave a value of 7.6×10^4 cells/ml ($8 \times 10^4 < \text{cells/ml} < 3.1 \times 10^5$) in aerobic and a low value of 8.5 cells/ml ($2 < \text{cells/ml} < 3.5 \times 10^1$) in anaerobic conditions. Methylophilic bacteria decreased after four days in aerobic incubation. Bacteria belonging to Beta and Gamma subclasses were detected only in aerobic conditions. Sulfate reducing bacteria were found in anaerobic cultures and in the presence of oxygen as well, forming aggregates. Bacteria belonging to the genus *Pseudomonas* were not detected with specific probe. RFLP analyses showed some similar patterns between the aerobic and the anaerobic enrichments. An involvement of the same microorganisms in aerobic and in anaerobic conditions for the degradation of aromatics monomers is suggested.

Introduction

Salt Pond is a shallow semi anaerobic basin, with a deep of about 5.5 m, seasonally chemically stratified, located in Woods Hole, MA (Wakeham, 1987). While aerobic processes dominate the epilimnion, the anaerobic hypolimnion generally has high concentrations of H₂S. This concentrations rise up to 5 mM in summer, in reason of the sulfate reduction in the bottom waters and sediments (Wakeham, 1984; 1987). This coastal basin receive input of fresh waters and marine waters, organic material containing lignin and cellulose residues is present. Lignin is a complex, three dimensional aromatic polymer containing also low molecular-weight phenolic compounds (Borneman et al., 1986). One of the most studied model of degradation of this complex natural polymer is the manganese peroxidase in *Phanerochaete chrysosporium* (Tuor et al., 1992). *Pseudomonas* is a diffused genus able to degrade aromatic compounds (Chapman and Ribbons, 1976). A microbial activity in soil enriched with preparation of AS-lignin and lignofulvonic acid was detected and an increase in the incidence of bacteria utilizing vanillin, syringic acid or protocatechuic acid was found (Kunc and Rybarova, 1977).

A *Clostridium thermoaceticum* strain and other acetogenic bacteria were able to use the methyl group of aromatic methyl ethers as a carbon and energy source (el Kasmi et al., 1994). A new strategy of anaerobic degradation was found in a denitrifying bacterium *Azoarcus anaerobius* LuFRes1, which is able to grow in the presence of resorcinol as sole carbon and energy source, involving an oxidative step for destabilization of the aromatic ring (Philipp and Schink, 1998). A similar pathway of degradation was found also in *Thauera aromatica* AR-1 in the anaerobic degradation of α -resorcylyate (Gallus and Schink, 1998).

The aim of this work was to study if microorganisms from Salt Pond sediments were able to grow in the presence of aromatic monomers compounds as carbon and energy source, in aerobic and anaerobic conditions. The characterization of the microorganisms was a goal of this work as well, to

detect if the same microorganisms were involved both in aerobic and in anaerobic conditions. Role of denitrifying bacteria in the degradation of these compounds by switching their own metabolism from the aerobic to the anaerobic conditions was tentatively analyzed.

Materials and Methods

Sampling

Sediment samples were taken from Salt Pond close to Woods Hole, MA from the upper 10 cm of a shallow nearshore region (about 0.5 m deep). The samples were collected in plastic buckets and stored in laboratory with a 2-3 inch covering layer of Salt Pond water, to maintain anoxic conditions in the sediments, and capped with aluminum foil.

Most Probable Number (MPN)

Three series of eight tubes each were filled with 9 ml of mineral medium in aerobic and three series in anaerobic conditions. Serial dilution from the sediment were carried out in each different series and tubes were incubated at 30°C. Turbidity was detected after 6 days and values were detected with MPN tables (American Public Health Association, 15th ed.).

Enrichment cultures

Enrichment cultures were carried out in Widdel Pfenning modified mineral medium (Widdel and Bak, 1992). The aerobic enrichment was carried out in a 1 L enermheier flask, the anaerobic in a vials of 1 L of volume. Different substrates were added to the mineral medium at a concentration of 1 mM: syringic acid (Sigma), salicylic acid (Sigma), benzoic acid (Sigma), vanillic acid (Sigma) at a concentration of 1 mM. NaNO₃ were added at a concentration of 4 mM. Aerobic and anaerobic cultures were incubated at 30°C. Aliquots of 9 ml were harvested at different times from each cultures to carry out different analyses.

Enrichments for homoacetogenic bacteria

Enrichments for homoacetogenic bacteria were carried out in mineral reduced medium (Widdel and Bak, 1992), in the presence of H₂/CO₂ mixture as carbon and energy source, bromoethanesulfonate (BES) at a concentration of 10 mM was added to the media in order to avoid growth of methanogenic bacteria. The media was distributed in vials of 50 ml of volume, with rubber cap with head space, and inoculated with 1 ml of sediment. When culture showed growth, a secondary enrichment was carried out in the same condition of growth. Two agar shakes processes were then carried out in order to isolate single colonies (Microbial Diversity Course 1998).

High Pressure Liquid Chromatography (HPLC)

Samples harvested from aerobic and anaerobic cultures at different times were stored at -70°C, then analyzed by HPLC (Water TM LC Model 1 plus). Twenty microliters of each samples and of different standards (salicylic acid, syringic acid, benzoic acid, vanillic acid 1 mM), were analyzed in a HPLC equipped with a Waters NovaPak C18 reversed-phase column. Samples were eluted in isocratic conditions, using 50% of distilled water with 0.1% of H₃PO₄ and 50% of methanol. The flow rate was 0.5 ml/min, the UV detection wavelength 210 nm, and the column oven was at room temperature.

Fluorescence In Situ Hybridization (FISH)

Samples for *in situ* hybridization were treated as suggested during the Microbial Diversity Course 1998. Probes used were the following: Universal (5'acggcggtgthtrc3'), Beta-proteos (5'tcactgctacacgyg3'), Gamma-proteos (5'ctttgcarccact3'), sulfate reducers (5'cgygcgcrtctytact3'), *Pseudomonas* sp. (5'ccttctccaact3') (Microbial Diversity Course 1998).

DNA extraction

DNA was extracted according to the method given in the Microbial Diversity Course 1998. Extracted DNA was then purified by using the Wizard purification system (Zhou et al., 1996).

PCR

The PCR reaction mixer was composed by 5 µl of 10X buffer, 5 µl of MgCl₂ 25 mM, 4 µl of dNTP's mix 2.5 mM, primers volume according to the reaction, template 1 µl, 1 TaqBead polymerase (Promega) or 0.2 µl of taqGold (Roche), according to the method, and dH₂O to give a final volume of 50 µl. PCR products were visualized in agarose 0.8% gel, stained with GelStar stain nucleic acid stain. Bacterial 16S rDNA Universal Primers: Forward position 8-23 (2µl) (AGAGTTTGATYMTGGC), Reverse position 1492-1475 (2 µl) (AGAGTTTGATYMTGGC). The annealing temperature was 55°C.

Checkerboard hybridization

After extraction of DNA, digoxigenin-labeled 16S rRNA amplicons were generated from aerobic and anaerobic cultures DNA, by using Dig Universal Forward position 8-23 (2 µl) (LAGAGTTTGATYMTGGC), Universal reverse primers position 1492-1475 (2 µl), and by running the reaction with a 50°C annealing temperature. PCR products were visualized by running 0.8% agarose gel stained with GelStar nucleic acid gel stain (FMC BioProducts). PCR products were then used for checkerboard hybridization (Microbial Diversity Course 1998).

Cloning

Amplified bacterial 16S rDNA samples from the two different enrichments, after 3 days for the aerobic and after 6 days for the anaerobic one, were cloned using TA TOPO cloning kit (Invitrogen) into *Escherichia coli*. Inserts present in white colonies were detected by PCR, amplifying the insert with 1 µl of TOPO-for (CCACTAGTASCGGCCGCC) and 1 µl of TOPO-Rev (CGGCCGCCAGTGTGATG) primers added to the mastermix containing 5 µl of 10X buffer (Roche), 5 µl of MgCl₂ 25 mM, 4 µl of dNTP's, 0.2 µl of Taq (Roche) and distilled filtered water to a final volume of 50 µl. PCR products were visualized in agarose gel 0.8% stained with GelStar stain nucleic acid stain.

Restriction Fragment Length Polymorphism (RFLP)

Sixteen different PCR products were analyzed for the anaerobic experiment and other sixteen for the aerobic one. Ten microliters of PCR product where the insert was present, were added to 10 µl of a mastermix buffer containing 2 µl of buffer n° 2, 0.1 µl of the restriction enzyme *Hin*PI, 0.05 µl of the restriction enzyme *Msp*I, and 7.8 µl of filtered dH₂O. The mixture was incubated overnight at 37°C. Reaction products were visualized in a 1.5% Methaphor-agarose gel, stained with GelStar staining.

Identification of isolated colonies

Isolated colonies sequencing was carried out by the Forsythe Dental Center. The phylogenetic placement of the 16S rRNA sequences was determined with the BLAST program.

Results

MPN counts

MPN analyses carried out in mineral media in the presence of different aromatic compounds, with and without oxygen, gave a value of 7.6×10^4 cells/ml in aerobic conditions ($8 \times 10^4 < \text{cells/ml} < 3.1 \times 10^5$), and in anaerobic cultures microbial growth reached a value of 8.5 cells/ml ($2 < \text{cells/ml} < 3.5 \times 10^1$).

Aerobic and anaerobic enrichments

Aerobic enrichments in the presence of different aromatic monomers showed the depletion of the concentrations of the aromatic compounds. After 3 days of incubation the aromatic compounds were completely depleted (fig. 1). The control with the same aromatic compounds added to the mineral medium showed only a low decrease in the concentration values of the different compounds (fig. 2).

Anaerobic enrichment showed a slow growth of microorganisms, degradation started after 8 days of incubation with a decrease in the concentration of the aromatic monomers compounds (fig. 3).

Fluorescence in situ hybridization (FISH)

Fluorescence *in situ* hybridization analyses showed hybridization by using the universal probe (fig. 4a), with the probe for sulfate reducing bacteria, both in aerobic and in the anaerobic samples harvested at different times (fig. 4b and 4c). With the other probes tested with samples from aerobic and anaerobic condition of growth, only the Beta fluorescent probes showed positive hybridization in the aerobic experiment (fig. 4d). No signal was found neither with *Pseudomonas*, nor with Gamma probes.

Checkerboard hybridization

Betas-all and enterics/some Gammas probes gave positive signal only in the experiment with oxygen, with a strong signal after two days of incubation at 30°C. Methylotrophic bacteria signal decrease with time in the samples collected from the aerobic experiment; no methylotrophic bacteria were found in the enrichment in anaerobic conditions. Alphas-all bacteria were present in aerobic and in anaerobic conditions, in the latter the signal is low, as well with the universal probe. SRB2 and SRB, some deltas, gave positive signal both in aerobic and in anaerobic conditions. Probes for low GC Gram-positive, *Planctomyces* and *Spirochetes* showed hybridization with all the samples, more evident in the aerobic conditions after 3 days of incubation (fig. 5).

RFLP analyses

RFLP analyses of the anaerobic sample (fig. 6a) showed two groups of similarities, one formed by five patterns, the other one by four patterns, the others patterns were different. In the presence of oxygen (fig. 6b), seven of the sixteen samples were grouped in one cluster, whereas the others patterns did not showed similarities. Similarities were found between patterns present both in aerobic and in anaerobic conditions.

Identification of isolated colonies

The colony harvested from the anaerobic enrichment in the presence of H₂/CO₂ and BES, was tentatively identified. The PCR product with the universal primers for 16S rRNA were sequenced, showing the 100% of similarities with the sulfate-reducing or sulfur-reducing dissimilatory bacteria *Desulfomicrobium apsheronum* (fig. 7).

Discussion

In aerobic enrichments with sediments from Salt Pond, aromatic compounds concentrations decrease due to the microbial activity. These compounds can also be oxidized (Schink, personal communication), anyway in the aerobic control no changes in the aromatic monomers concentrations were detected, suggesting a direct involvement of microorganisms in the degradation of these compounds. In the anaerobic experiment, growth started later, microbial growth values detected by MPN were rather low. This value were detected after 6 days, and in the experiment turbidity increases and degradation were observed after 8 days of incubation. An increase in the concentration of benzoic acid was found in anaerobic conditions, probably because it represents an intermediate in the degradation pathway of salicylic acid.

Degradation of aromatic compounds is frequently carried out by bacteria belonging to the genus *Pseudomonas* (Chapman and Ribbons, 1976), in this experiments the presence of this genus was suggested by checkerboard hybridization with the Gamma probe, but not by *in situ* hybridization experiments with the fluorescent *Pseudomonas* probe. The method of checkerboard hybridization is probably more specific, on the other hand it is also possible that in this experiment others microorganisms different from the *Pseudomonas* genus are able to degrade the aromatic compounds. Sulfate reducing bacteria were detected in the two enrichments, in aerobic conditions they are present in clusters, attached to the sediment particles, probably to find the lowest concentrations of oxygen. The decrease of the methylotrophic bacteria showed a changes in the culture with time, probably the disappearance of the right substrates of growth. RFLP analyses showed some similarities among the patterns in aerobic and in anaerobic conditions. This behavior suggest an involvement of the same microorganisms able to degrade aromatic compounds in aerobic and in anaerobic conditions. Denitrifying bacteria are probably involved in this degradation and they can probably switch their own metabolism from the aerobic to the anaerobic conditions (Philipp, personal communication).

The identification of a colony isolated from an enrichment for homoacetogenic bacteria showed a similarity of 100% with the sulfate-reducing or sulfur-reducing dissimilatory bacteria *Desulfomicrobium apsheronum*. Due to the isolation carried out from an agar shake plate, the presence of this unexpected species can be caused by a wrong way to work and a contaminant was present. On the other hand, this species is not well known and can probably switch from the use of sulfate as electron acceptor to the use of CO₂, following the metabolism of the homoacetogenic bacteria (Schink, personal communication).

The presence of sulfate reducing bacteria is confirmed by different analyses, giving us important information about the importance of these microorganisms in the Salt Pond sediments probably also in the degradation of natural aromatic compounds.

Acknowledgments

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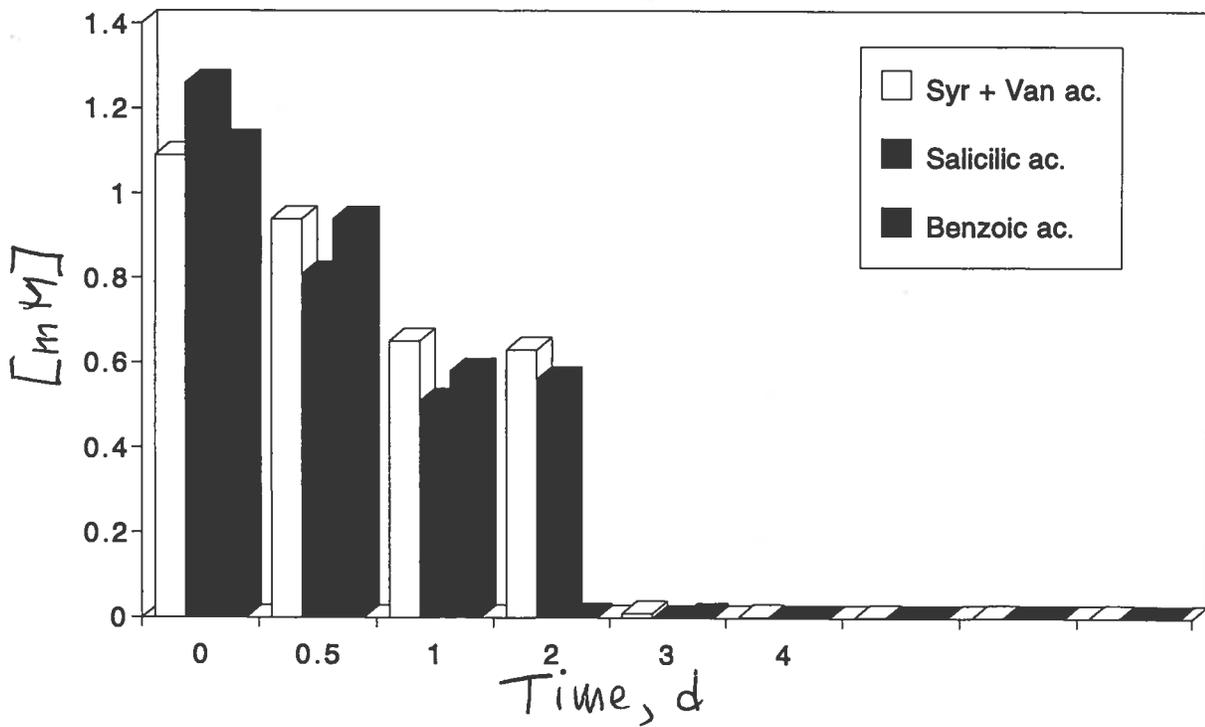


Figure 1 - Aerobic enrichment, concentration of aromatic compounds were detected by HPLC analyses.

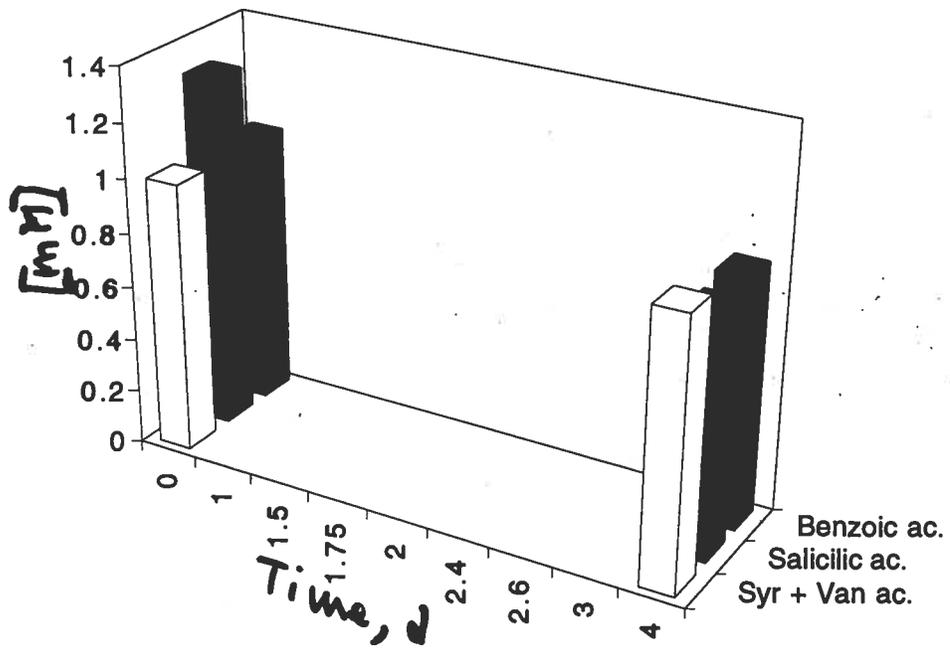


Figure 2 - Control in mineral media and anaerobic compounds in aerobic conditions.

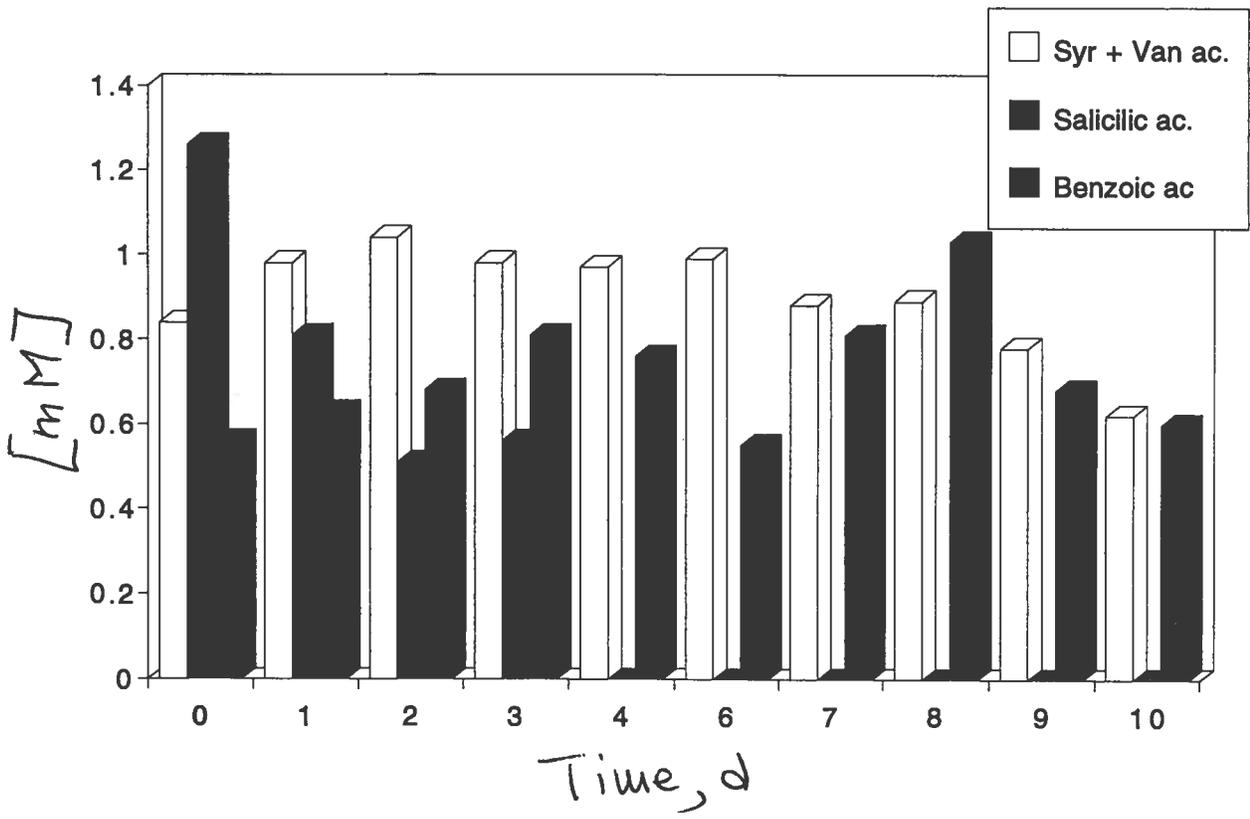


Figure 3 - Anaerobic enrichment, concentration was detected with HPLC analyses.

Fig. 4

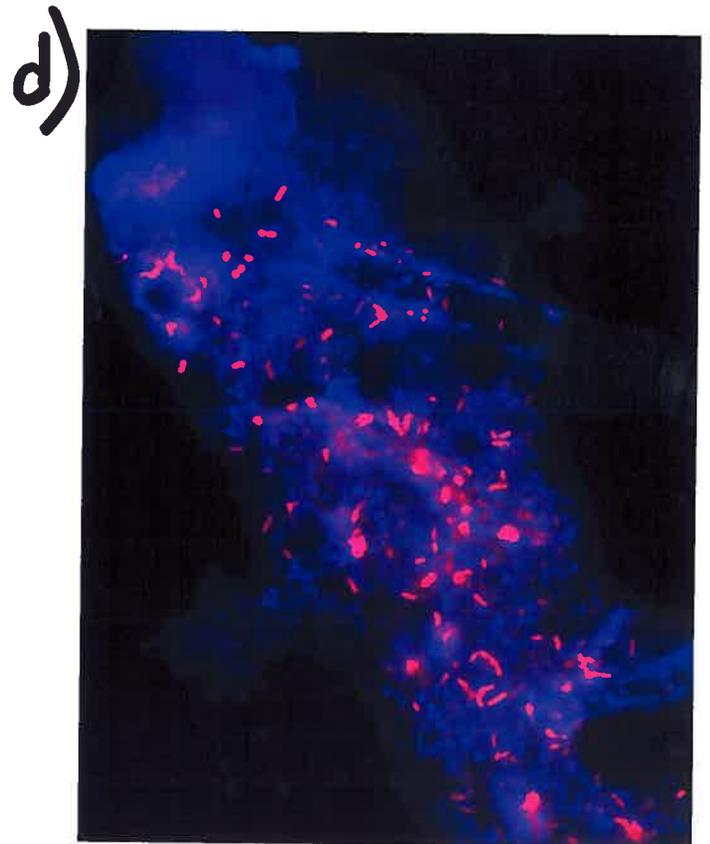
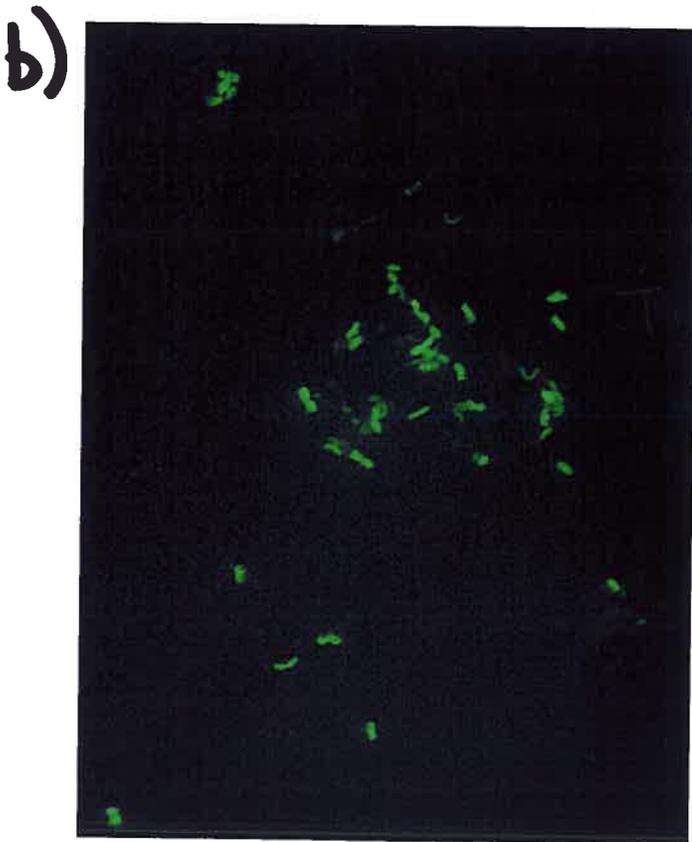


Figure 4 - Fluorescent *In Situ* Hybridization with different probes: Gamma probe a); Universal probe b); sulfate reducing bacteria probe c, d)

(% Difference)

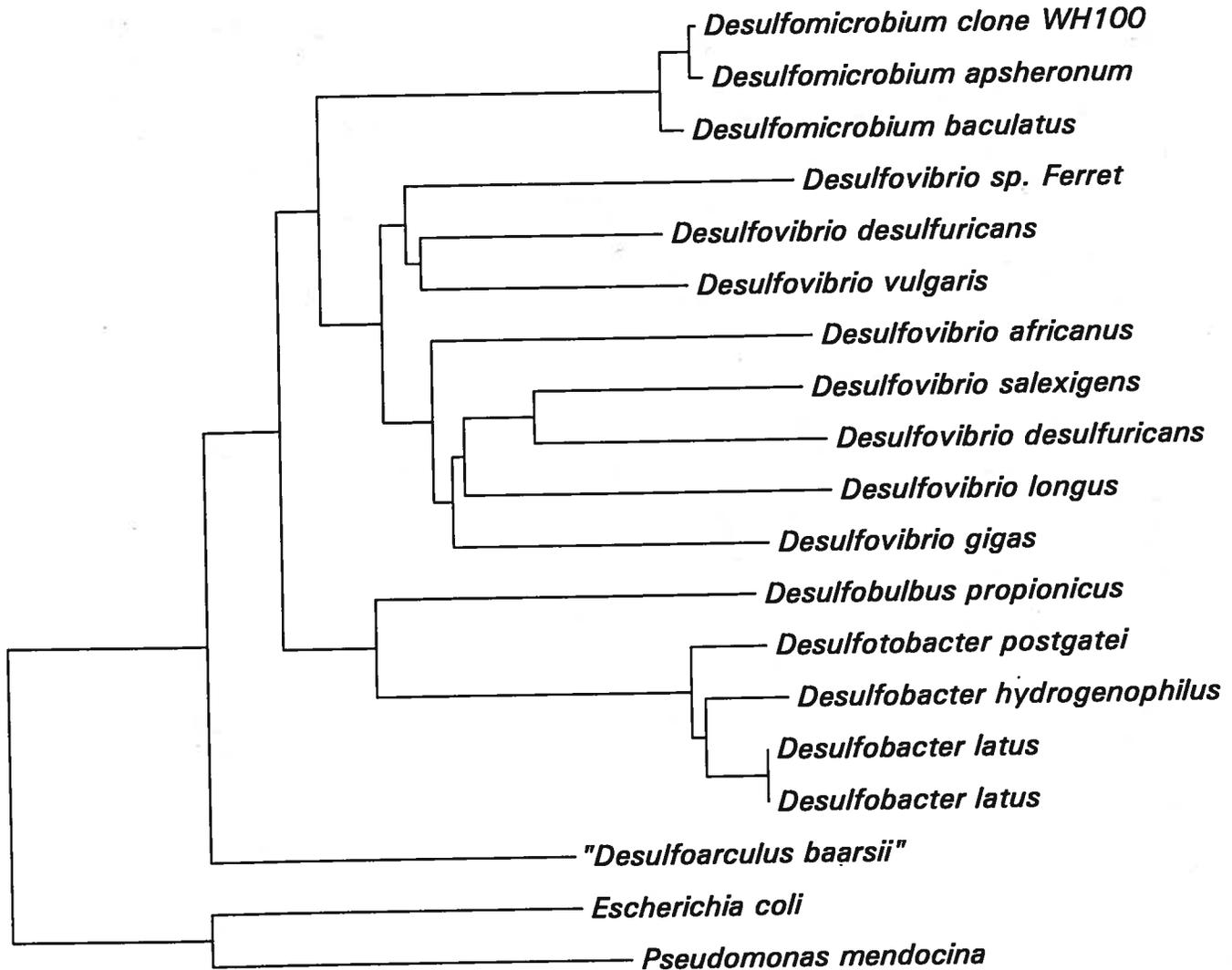
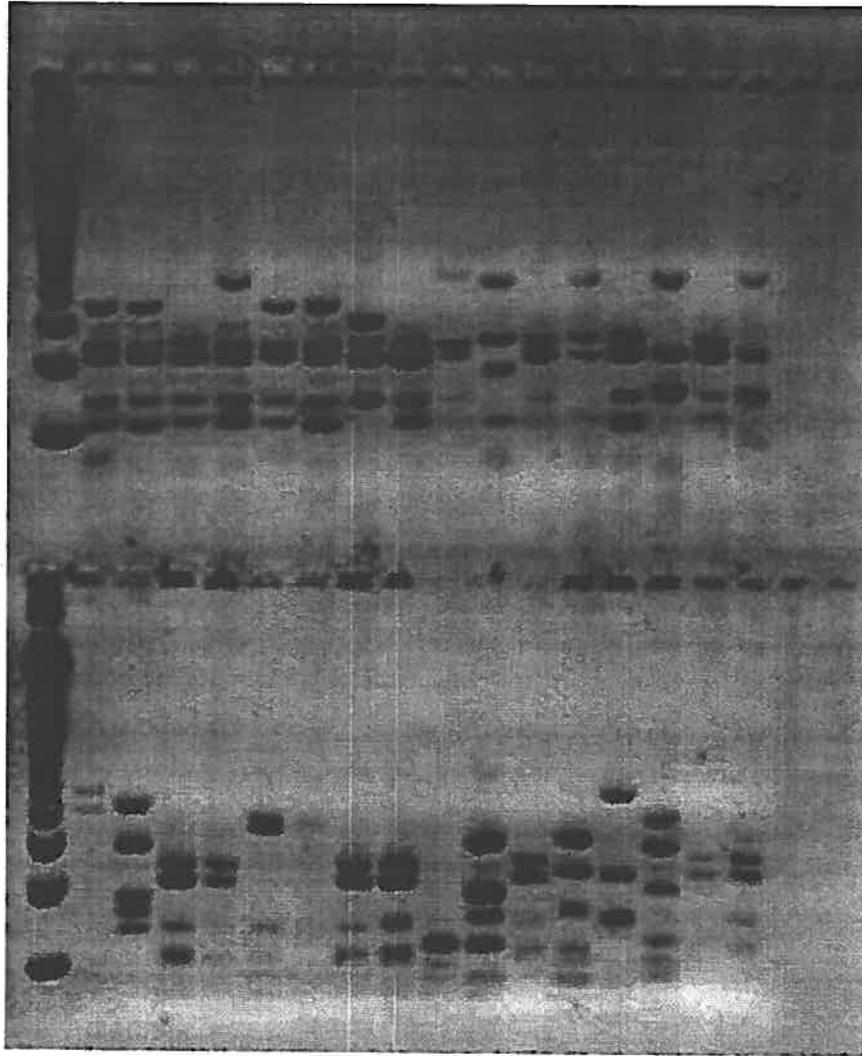


Figure 7 - Phylogenetic tree after 16S rRNA amplification with universal primers.

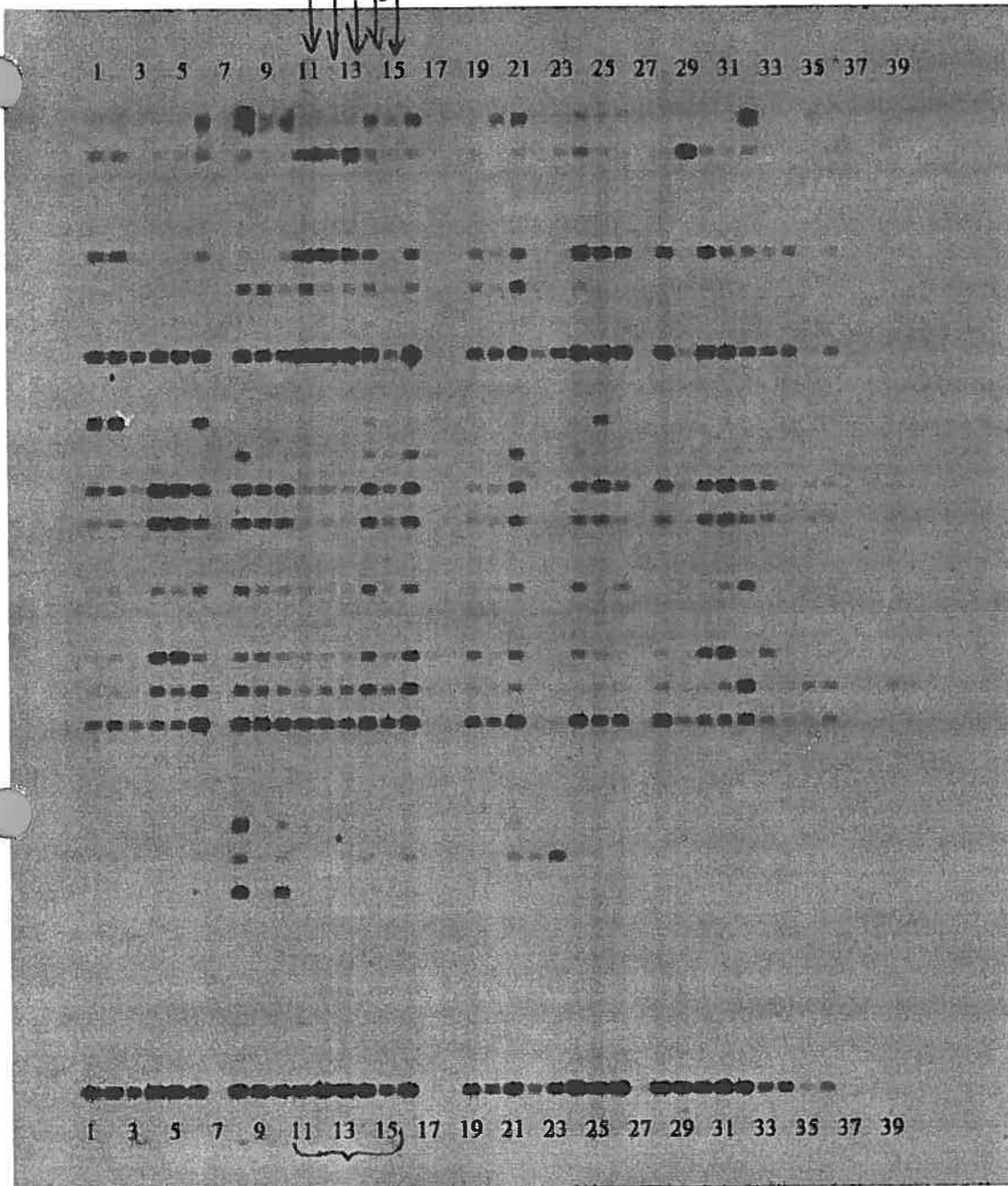


-O₂
t₆

+O₂
t₄

Figure 6 - RFLP analyses: upper: anaerobic experiment; bottom: aerobic experiment.

$t_0 + O_2$
 $t_1 + O_2$
 $t_2 + O_2$
 $t_4 + O_2$
 $t_6 - O_2$



- Berry "Purple Sulfur "
- Betas-all
- Nitrosolobus/Nitrosovibrio
- Nitrosomonas spp
- Enterics / some gammas
- Methylotrophic bacteria
- ANG Alteromonas sp.
- Alphas-all
- ANG Shewanella sp.
- Vibrio gazogenes
- Berry "Plastid"
- SRB2, some deltas
- SRB, some deltas
- Berry SRB clones C16/C18
- LoGC Gram Positive
- Green Sulfur-all
- Planctomyces-all
- Spirochetes-
- Flavos-all
- Bacteroides-
- Cytophaga berry C10
- Cytophaga berry C9
- Cyanobacteria
- Microcoleus sp.
- Lyngbia sp.
- Nostoc sp.
- Oscillatoria sp.
- Phormidium ectocarpi .
- Synechococcus sp.
- Universal

Key:

- | | | | |
|------------------------------|--------------------------------------|--------------------------|-------------------------|
| 1. Silvana-salt pond 1 | 12. Milva enrich $t_2 + O_2$ | 23. Karin Cyan. Pure | 34. Yoshiko- H_2/Fe |
| 2. Silvana-salt pond 2 | 13. Milva enrich $t_4 + O_2$ | 24. Scott- Begg. Environ | 35. Pond Berry |
| 3. John oyster | 14. Milva enrich $t_6 + O_2$ | 25. Andreas Deep Sea | 36. Pk sand berry 1/10 |
| 4. Group I- ED | 15. Milva enrichment t_6 w/o O_2 | 26. Andreas Deep Sea | 37. Pk sand berry 1/100 |
| 5. Group I-LD | 16. Grp II sediment | 27. Andreas [no good] | 38. Brown berry |
| 6. Group I-MD | 17. Grp II sediment [No good] | 28. Andreas Deep Sea | 39. Pink sand 1/10 |
| 7. Group I-G1 [No good]] | 18. [No good] | 29. Yoshiko ThioS | 40. Mussel 1/10 |
| 8. Group I-P2 | 19. Patti Sip. Hi Nitrogen plot | 30. Yoshiko- H_2/Fe | |
| 9. Group I-G3 | 20. Patti Sip. Low Nitrogen plot | 31. Grp II-PSB | |
| 10. Group I-B4 | 21. Patti Sip. Control plot | 32. Grp II Cyto/PS | |
| 11. Milva enrich $t_0 + O_2$ | 22. Karin Cyan. Assoc. | 33. Grp II SRB | |

Figure 5 - Checkerboard hybridization analyses, line 11: time 0, line 12: time 1 + O_2 ; line 13: time 2 + O_2 ; line 14: time 4 + O_2 ; line 15 time 6 - O_2 .

A C-type Cytochrome act as an Electron Carrier for Electron Transfer to Iron(III) hydroxide

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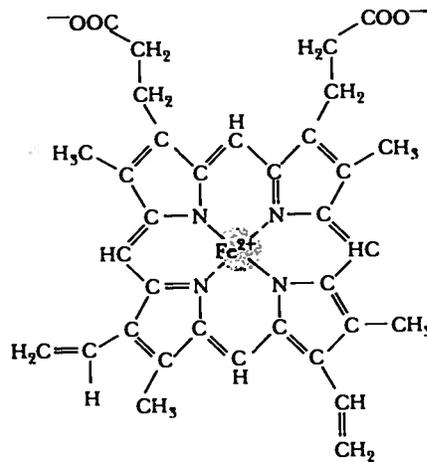


Figure 8.13 The heme portion of a cytochrome molecule. The tetrapyrrole is also called a porphyrin. The heme is attached to a protein through the ethylene groups at the bottom of the molecule.

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