Molecular and microbiological approaches to study sulfate-reducing bacteria in a stratified coastal salt pond

Silvana Tarlera Robles

Microbial Diversity 1998
Woods Hole, MA

Universidad de la República
Montevideo, Uruguay
starlera@bilbo.edu.uy

ABSTRACT

Sulfate-reducing bacteria could be successfully distinguished from other bacteria present in the sediment sample with a specific rRNA-targeted probe. Furthermore, DAPI staining of the samples probed with a universal probe, confirmed that the fluorescent-labeled probe assay was sensitive enough to reveal the presence of most cells. High amounts of rRNA detected in the subsample suggests that most of the bacteria were active.

The RFLP patterns obtained showed an important diversity among the 16S rRNA genes retrieved from the sediment supernatant. Species belonging to the *Cytophaga- Flexibacter- Bacteroides* division were detected in the agar shake dilutions. Direct DNA extraction from the sediment supernatant revealed the presence of the following strains: *Desulfobacter* sp., a sulfate-reducer belonging to the delta subdivision of Proteobacteria; *Thiomicrospira* species belonging to the gamma subdivision of Proteobacteria where they form a distinct monophyletic lineage and an *Arcobacter* strain belonging to the epsilon subdivision which also contains other spirilloid sulfur-reducers. Also a non-identified bacterium with 91% similarity to an unknown deep sea clone was recovered.

The present work suggests that the combined application of molecular techniques, which do not require selective cultivation, with physiological and traditional approaches in marine microbiology offers an advantage to obtain a better insight in the identification and quantification of individual microbial species in naturally occurring microbial communities.

INTRODUCTION

In marine anoxic ecosystems, the mineralization of organic matter occurs through a series of microbial metabolic reactions that produce increasingly less complex organic compounds where the end products of one group of reactions are generally utilized as substrates by another group of organisms. Sulfate is normally present in high amounts in marine sediments. Therefore, after oxygen is depleted by aerobic respiration, sulfate respiration becomes a dominant process.

The aim of this project was to study the presence of sulfate-reducing bacteria combining molecular and microbiological methods in a marine environment. Our location was a shallow eutrophic marine basin known as Salt pond near Woods Hole that exhibits density stratification where an anoxic zone has been described to rise to within 2-3 m of the pond surface. The combined application of molecular techniques, which do not require selective cultivation, with physiological and traditional approaches in marine microbiology offers an advantage to obtain a better insight in the identification and quantification of individual microbial species in naturally occurring microbial communities.

The use of gene probes for selected groups of bacteria makes it possible to observe and quantify them directly in environmental samples. These probes are designed with the 16SrRNA as a target and require metabolically active cells in order to be visualized since only cells with high levels of ribosomal RNA can be detected. Direct isolation methods were performed for the enumeration and isolation of sulfate-reducers as well as selective enrichment strategies in combination with *in situ* hybridization with domain and group specific probes and 16S rRNA sequence analysis of the population. The presence of moderately thermophilic sulfate-reducing activity was also investigated.

MATERIALS AND METHODS

Sampling procedure. Sediment was collected from Salt Pond from an aproximate depth of 1m near the shore and stored at 10 °C until it was inoculated the next day. The sediment decanted and the sample was divided in two parts: the black sediment and the aqueous supernatant on top.

Microscopy. *In situ* hybridization procedures were performed according to the Microbial Diversity Course 1998 guide.

Direct isolation. The agar shake dilution technique as described by Pfennig, 1978 was employed to perform direct isolation of SRB. Two substrates were tested lactate, acetate and ethanol and serial dilutions up to 10^{-7} were carri d out in saltwater media. Sulfide-reduced media was prepared after Widdel and Bak (1992).

Enrichment cultures. Enrichment cultures for SRB were carried out in liquid saltwater and fresh water media prepared after Widdel and Bak (1992). Ethanol, acetate and lactate served as independent substrates. Two incubating temperatures were tested 28 °C and 55 °C. Sulfate concentration was 20 mM in all enrichments.

Genomic DNA extraction and PCR amplification of 16S rRNA gene sequences; construction of a 16S rRNA gene clone library and RFLP analysis.

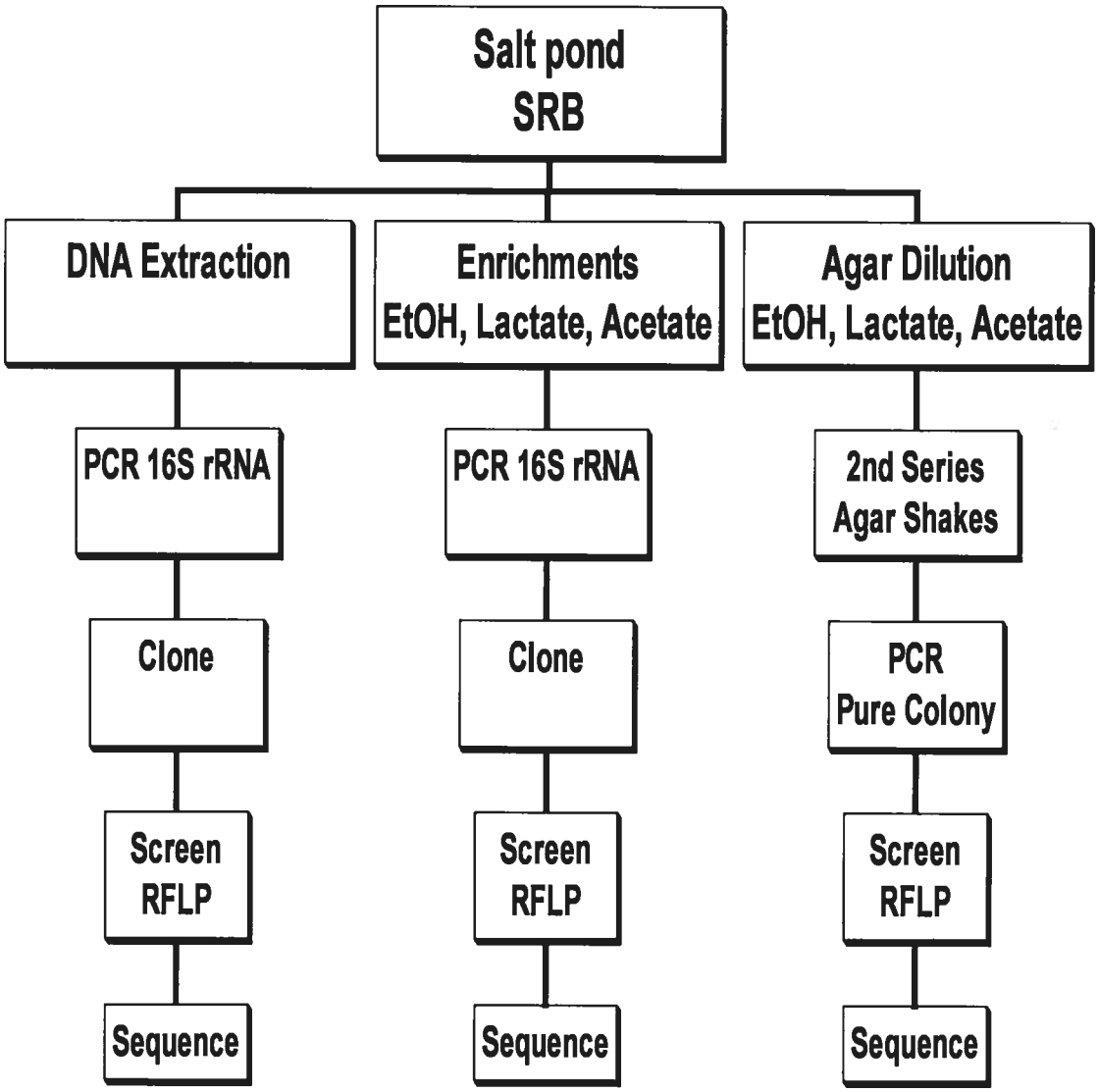
Phenyl:chloroform:isoamyl alcohol mix / extraction buffer was added to the sample together with glass beads in a bead beater tube and bead beat on a mini bead beater for one minute. Polymerase chain reaction (PCR) was done using an established protocol for the Microbial Diversity course. Amplification of the 16s rRNA gene was done using the primers 8-forward and 1492-reverse. Taq polymerase was added in wax bead form (Promega). Gels were run in 0.8% agarose prepared in 0.5X TBE buffer. If a strong band was visible, indicating the presence of the16s rRNA amplicon, cloning was done with either an Invitrogen Topo cloning kit, or an Invitrogen Blunt cloning kit. Approximately 24 clones were then examined for the 16s rRNA gene insert by a second PCR amplification. All of these PCR products were then restriction enzyme digested at 37°C for restriction fragment length polymorphism (RFLP) analysis following an established protocol developed for the Microbial Diversity course. Twenty microliter samples were loaded on a 1.5% low melt agarose gel, and band pattern differences were determined visually. Based on this analysis, it was decided which samples would be sequenced.

DNA sequencing and similarity analysis. rRNA genes from representatives of different categories of clones defined by RFLP were sequenced in an automated sequencing apparatus. The similarity analysis was performed using the program BLAST, which compares a given sequence to a 16S rRNA sequence database and returns a list of the most similar sequences found. The phylogenetic tree was constructed using the TreeCon program.

Checkerboard hybridization. The reverse-capture PCR based checkerboard hybridization was performed after the protocol handed out during the course. Briefly, capture probes were applied to Nylon membranes using Minislot, capture probes UV crosslinked to membrane and further digoxigenin-labeled PCR 16S rDNA amplicons hybridized with probes in Miniblotter. Chemifluorescence or chemiluminescence detection was applied finally.

HPLC Analysis. Fatty acids were detected and quantified in liquid medium using a Waters HPLC equipped with an UV detector (wavelength of 210 nm). The oven was operated isothermally at 60 °C. A Shodex column, Ionpak KC-811, was used for separation, and the eluent was 0.1% H_3PO_4 (flow rate of 1 mL/min). Samples were prepared by transferring 400 μL of medium into sterile 1.5 mL eppendorf tubes. The tubes were centrifuged for 10 minutes at 13,000 g. The supernatant was transferred to clean HPLC vial inserts for analysis. Standards (5 mM) were prepared and analyzed with the samples.

Strategy


Salt pond
SRB
DNA Extraction
Enrichments
EtOH, Lactate, Acetate
Agar Dilution
EtOH, Lactate, Acetate
PCR 16S rRNA
PCR 16S rRNA
2nd Series
Agar Shakes
Clone
Clone
PCR
Pure Colony
Screen
RFLP
Screen
RFLP
Screen
RFLP
Sequence
Sequence
Sequence

RESULTS

Microscopy. SRB enrichments were examined under phase-contrast microscopy with epifluorescence. DIC (Differential and Interference Contrast) microscopy allowed the observation of distinct straight and curved rod-shaped bacteria of different thickness (Fig.1a). A high amount of the bacteria were observed after *in situ* hybridization with a universal probe as compared to the amount of DAPI stained cells. Furthermore, a SRB specific fluorescent-labeled probe revealed the presence of this group of bacteria in the sample (Fig.1b).

Direct isolation. In one week, colonies were obtained up to the 10^{-5} dilution in the ethanol series while colonies were observed up to the 10^{-4} dilution on lactate and acetate. Purification was attempted through a second series of agar shakes from isolated colonies on these substrates. Colonies of different morphologies and colours were detected.

TABLE 1.

Enrichment (20 mM)	**Growth**	**Microscopy**	**FISH**	**VFA (mM)**
FW, Lactate **28 °C**	+	Straight and curved rods	-/+	5.6 Acetate 12 Propionate
FW, Ethanol **28 °C**	+	curved rods, vibrio-like motility, straight rods	+	5.0 Acetate
FW, Propionate **28 °C**	–	–	nd	–
SW, Lactate **28 °C**	+	spore-forming rods	–	2.1 Acetate 7.8 Propionate
SW, Ethanol **28 °C**	+	curved rods, vibrio-like motility	+	6.6 Acetate
SW, Ethanol **55 °C**	+/-	spore-forming rods	–	5.0 Acetate unknown peaks
SW, Lactate **55 °C**	–	–	nd	–

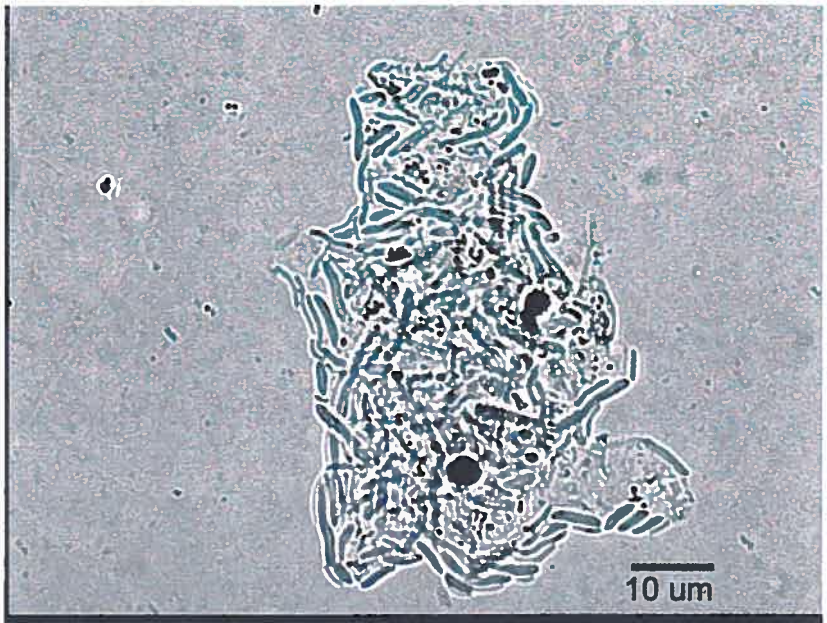


Fig. 1a. DIC

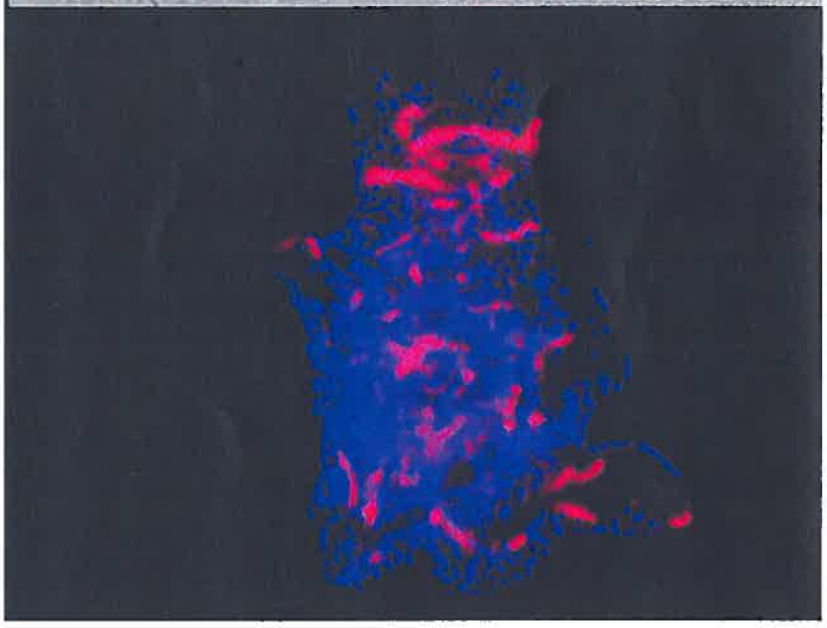


Fig. 1b. SRB probe

Estimation of molecular diversity. DNA was only successfully extracted from the aqueous supernatant on top of the sediment sample. Two dilutions were assayed and the best results were obtained with 1:10 dilution. DNA obtained was further purified through Wizard Columns before PCR amplification with universal primers. After blunt cloning of the PCR products and transformation, 24 colonies were chosen and checked for the insertion detected by PCR. About 50% of the clones tested positive for the insert of the correct size. A RFLP analysis was performed on 21 of the amplification products of the correct size as well as on 4 colonies obtained from the agar shakes. Several different restriction patterns were obtained (Fig. 2). The four colonies picked from the agar shake tubes gave different restriction patterns and were therefore chosen to be sequenced.

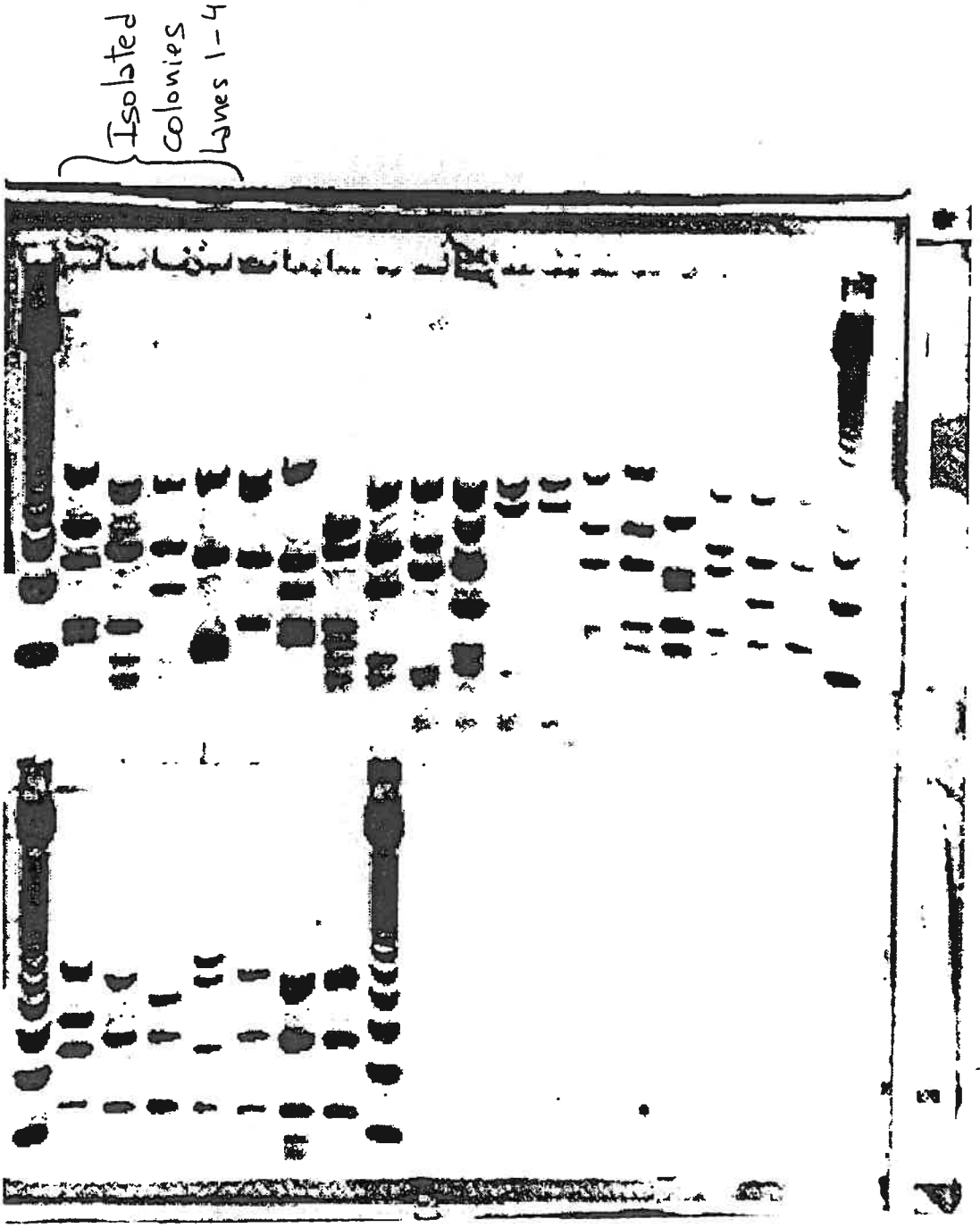


Fig 2. RFLP

Sequence similarity analysis. Seven unique clones together with the four colonies from the agar shakes tubes were selected on the basis of their different restriction pattern and were sequenced. The obtained sequences were about 500 bp in length. The sequences were then compared to a 16S rRNA sequence database and were analyzed for similarity to other known sequences. The four colonies obtained from the agar shake tubes were about 90% similar to *Cytophaga* species previously described. However, the four colonies seemed to be different species of the same genus. Of the seven other clones sequenced, four sequences referred to as WH73, WH77, WH78 and WH79, respectively, were found to be similar to sequences from four *Thiomicrospora* species isolated from deep-sea hydrothermal vents, with similarity ranges from 89 to 98%. The BLAST search for the other sequences revealed a 93% similarity to a *Desulfobacter sp.* isolated from salt marsh sediment, a 94% similarity to an *Arcobacter* strain isolated from a hypersaline cyanobacterial mat and a 91% similarity to an unidentified bacterium from deep-sea sediments.

Checkerboard hybridization results. Strong hybridization signals were obtained with the following probes: enterics and some gammas, alphas, vibrio, srb and some deltas and all flavos (Fig. 3). Weaker signals were obtained against all betas, low G+C gram positive, planctomyces, spirochetes and all-flavos. It is important to note that both the alphas and the flavos were not highly specific.

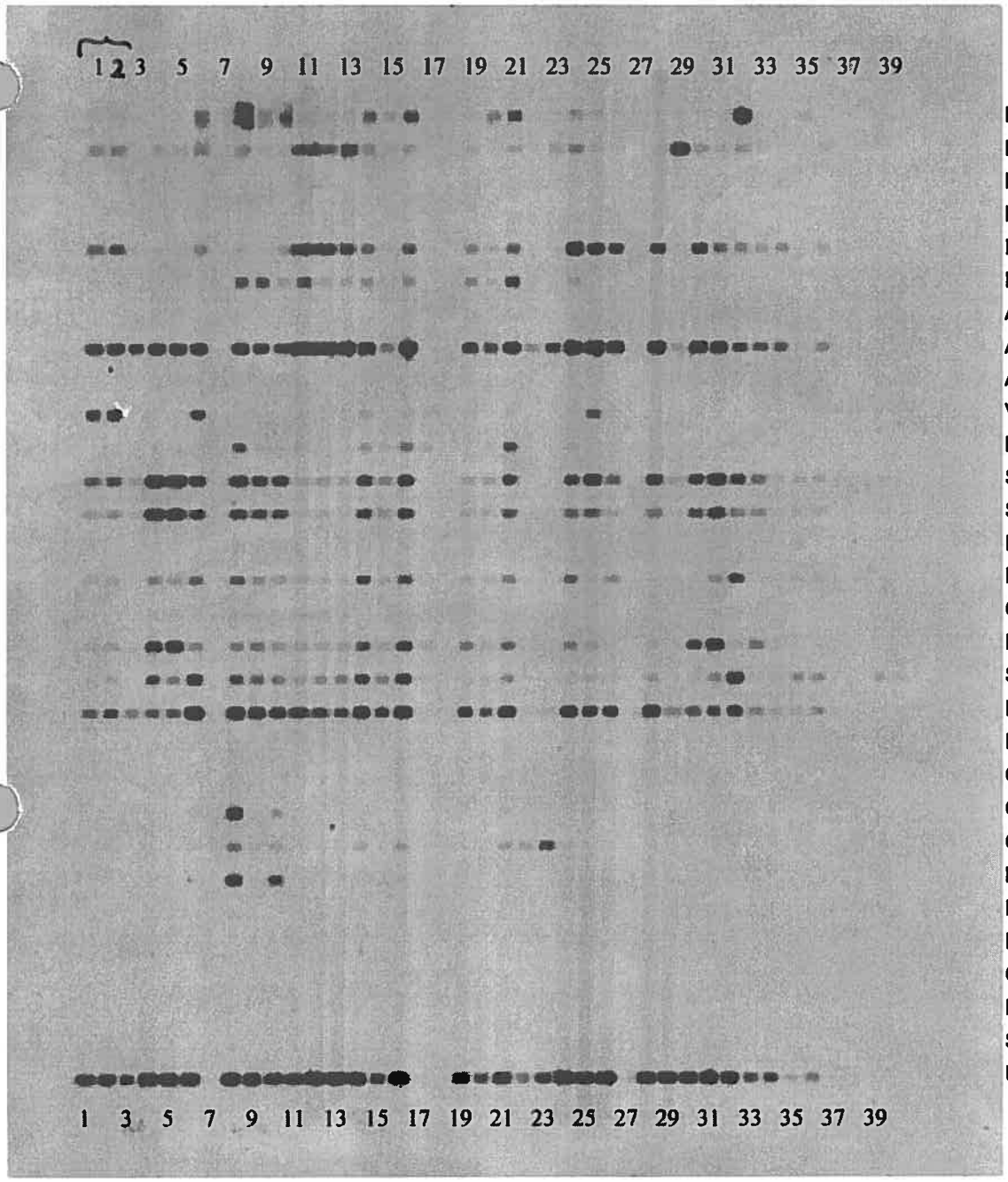


Fig 3. Lanes 1 and 2.

Key:

1. Silvana-salt pond 1
2. Silvana-salt pond 2
3. John oyster
4. Group I- ED
5. Group I--LD
6. Group I-MD
7. Group I-G1 [No good]]
8. Group I-P2
9. Group I-G3
10. Group I-B4
11. Milva enrich $t_0 + O_2$
12. Milva enrich $t_2 + O_2$
13. Milva enrich $t_4 + O_2$
14. Milva enrich $t_8 + O_2$
15. Milva enrichment t_6 w/o O_2
16. Grp II sediment
17. Grp II sediment [No good]
18. [No good]
19. Patti Sip. Hi Nitrogen plot
20. Patti Sip. Low Nitrogen plot
21. Patti Sip. Control plot
22. Karin Cyan. Assoc.
23. Karin Cyan. Pure
24. Scott- Begg. Environ
25. Andreas Deep Sea
26. Andreas Deep Sea
27. Andreas [no good]
28. Andreas Deep Sea
29. Yoshiko ThioS
30. Yoshiko-H_2/Fe
31. Grp II--PSB
32. Grp II Cyto/PS
33. Grp II SRB
34. Yoshiko-H_2/Fe
35. Pond Berry
36. Pk sand berry 1/10
37. Pk sand berry 1/100
38. Brown berry
39. Pink sand 1/10
40. Mussel 1/10

DISCUSSION

Sulfate-reducing bacteria could be successfully distinguished from other bacteria present in the sediment sample with a specific rRNA-targeted probe. Furthermore, DAPI staining of the samples probed with a universal probe, confirmed that the fluorescent-labeled probe assay was sensitive enough to reveal the presence of most cells. Most bacteria detected in the subsample were active since they contained high amounts of rRNA which is usually proportional to growth rate.

The RFLP pattern showed an important diversity among the 16S rRNA genes retrieved from the supernatant. After sequencing seven clones, six appeared to be different species. However, these results are preliminary and should be confirmed by sequencing the complete 16S rRNA gene, followed by performing an alignment and ending in the construction of a tree in order to obtain insight into the correct phylogenetic position of these clones.

The direct estimation of sulfate-reducers by a viable method using lactate, acetate and ethanol could not be accomplished due to the selection of bacteria belonging to the *Cytophaga* genus according to the sequence analysis. A phylogenetic tree confirms these clones cluster together as belonging to the *Cytophaga- Flexibacter- Bacteroides* division (Fig. 4.). It is important to take into account that due to time course limitations, it was only possible to PCR and sequence the DNA from colonies that appeared earlier in the lower dilutions. Thus, they might not be quantitatively important in this ecosystem. It must be remarked that no hybridization signal could be detected in the checkerboard experiment with two cytophaga species-specific probes. On the other hand, it has been suggested that these bacteria are ubiquitous and might play a major role in the turnover of organic matter in nature since they can adapt to low nutrient levels (Reichenbach, 1992).

Cytophaga Flexibacter Bacteriodes (CFB) Division

Microbial Diversity 1998

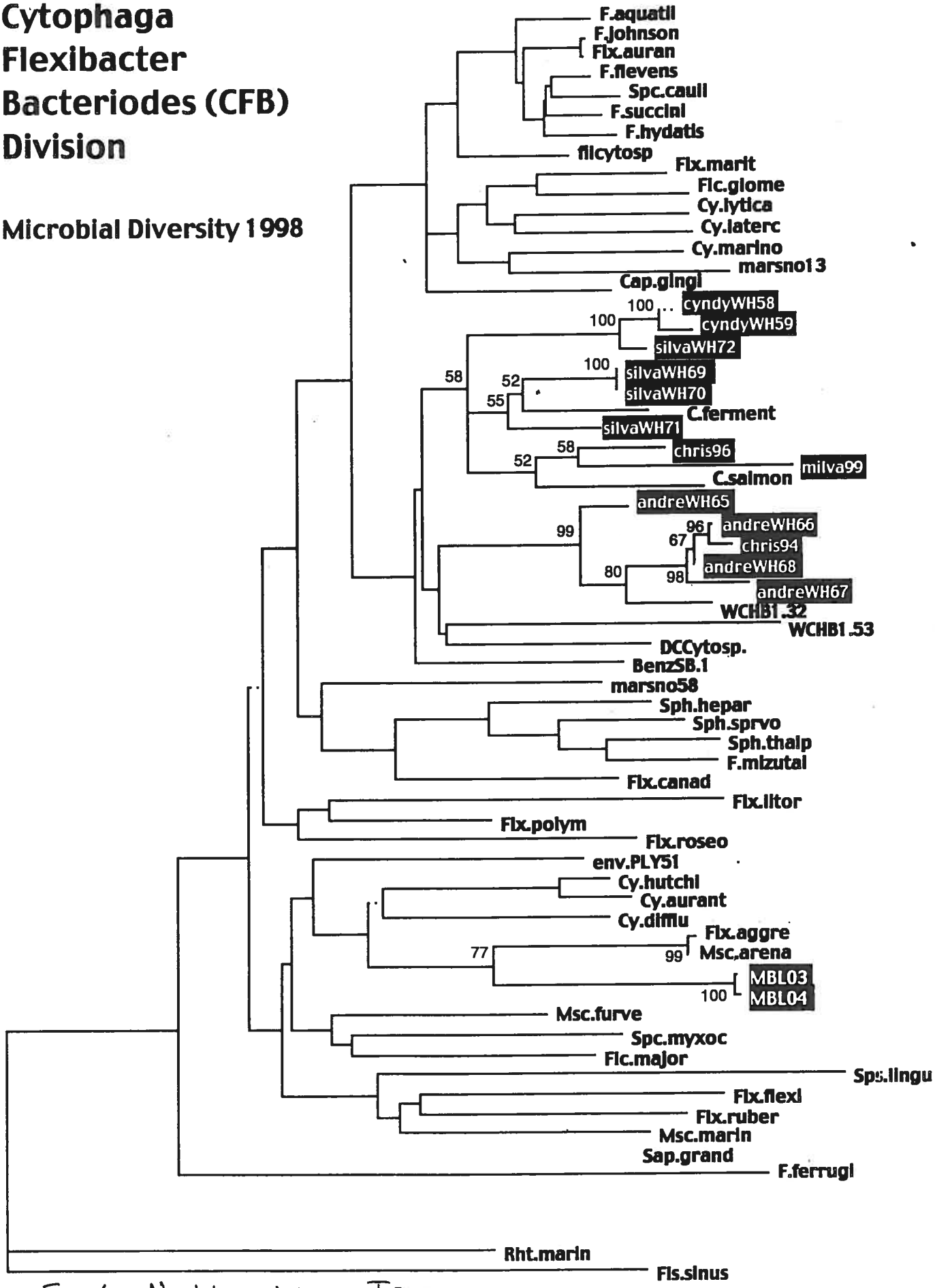


Fig. 4. Neighbor-joining Tree

Nevertheless, one of the clones recovered matched a *Desulfobacter* species. It is a sulfate-reducer which belongs to the delta subdivision of Proteobacteria which forms a distinct group of complete oxidizers within the rest of gram-negative sulfate reducers. Thus far, all species described within this genus have been reported to require high NaCl concentrations. To our knowledge, all spore-forming SRB known to date have been classified as belonging to the genus *Desulfotomaculum*. Therefore, it is highly likely that the 55°C spore-forming bacteria growing in the ethanol enrichment belongs to this genus. Most of these moderately SRB have been isolated from freshwater habitats and it would have been interesting to further characterize this enrichment. The poor growth observed in this enrichment could be attributed to the higher sensitivity toward sulfide than most Gram-negative sulfate reducers (Widdel, 1992).

Interestingly, four clones were similar to *Thiomicrospora* species isolated from deep-sea samples. They belong to the gamma subdivision of the class Proteobacteria where they form a distinct monophyletic lineage. They have been reported to be quite ubiquitous and have been isolated from such habitats as coastal sediment along the coast of Chile, microbial mat of the hypersaline pond in Egypt and saline springs in Germany (Brinkhoff, 1997). They are sulfur-oxidizing bacteria which might ecologically complement sulfate-reducing bacteria which act as the source of sulfide in these sulfide-producing hydrothermal vents (Muyzer et al., 1995). Since the clones were originated from the liquid supernatant on top of the sediment sub-sample it might well be that they play a role similar to that described in the hydrothermal vents. A phylogenetic tree was constructed using a distance matrix method (Neighbor-joining) and bootstrapped 100 times including the four sequenced clones (Fig. 5). Clones WH 73 and 78 clearly form a distinct branch and are different from *Thiomicrospira crunogena* which gave the highest similarity score obtained in the BLAST search. On the other hand, clone WH79 is probably a chimera.

Gamma Proteobacteria

Thiomicrospira Group

Salt pond

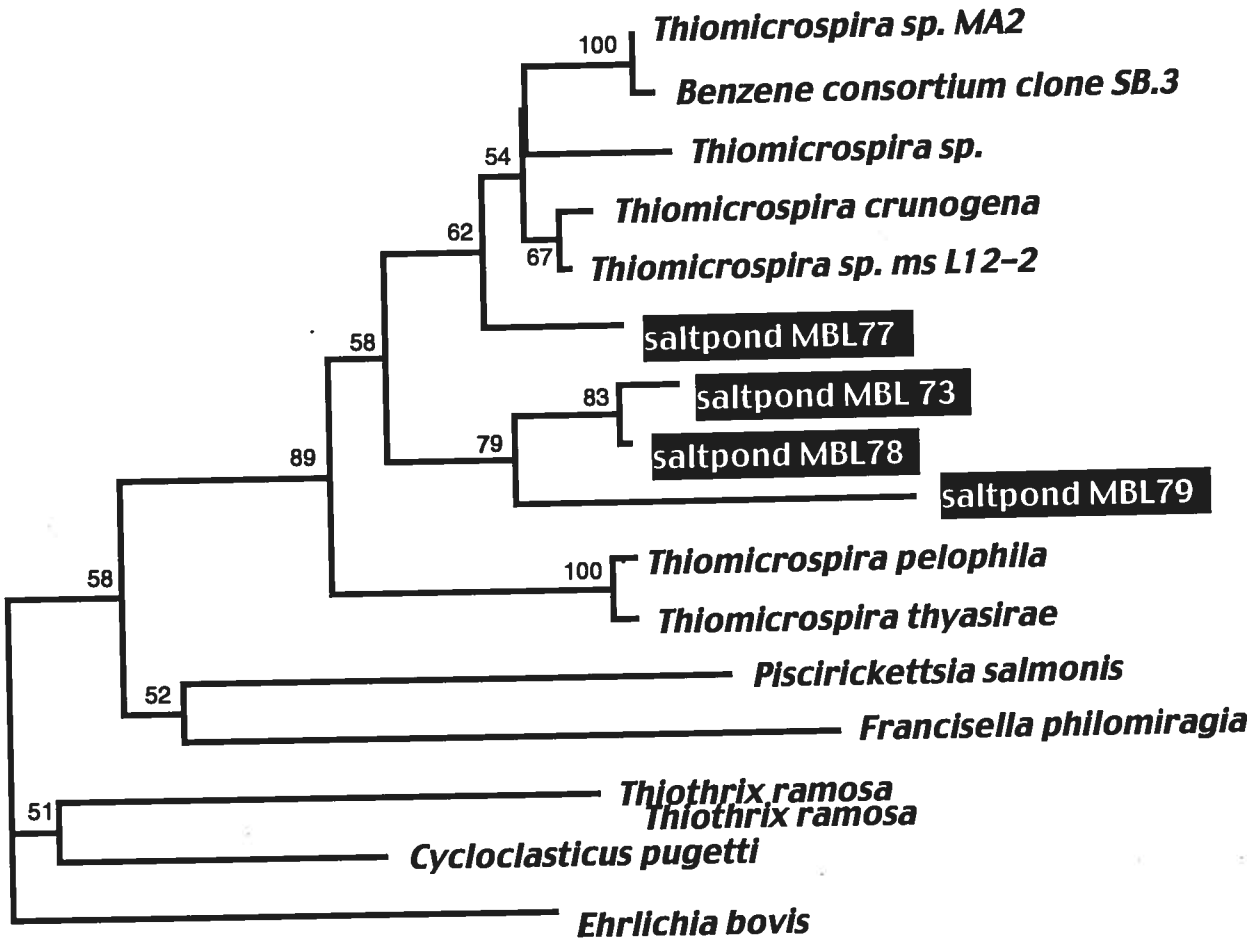


Fig. 5 Neighbor-joining tree.

NJ 100 bootstraps

Surprisingly, the other sequence retrieved from the supernatant was similar to an *Arcobacter* species which belongs to the epsilon subdivision of the class Proteobacteria which also contains the genera *Campylobacter, Wolinella, Thiovolum,* and *Helicobacter* and are known as spirilloid sulfur-reducers. Recently, a stable coculture of an *Arcobacter* strain and a *Desulfovibrio* strain obtained from a cyanobacterial mat surface layer, has been shown to be capable of sulfate reduction after exposure to oxic and microoxic conditions (Teske, A.,1996). In this report, the authors concluded that activity and growth of sulfate reducers in a habitat with large aerobic-anaerobic fluctuations probably depend on their close association and coculture formation with facultative aerobes. Also this previous report was an interesting example of how molecular identification of the two components of this coculture allowed the design of specific culture conditions to separate and isolate both strains in pure culture. This approach facilitates the combined molecular and physiological analysis of mixed cultures and microbial communities.

Finally, a genus or even a species-specific probe fluorescently labelled could be developed from these sequences in order to probe the original sample and quantify the relative abundance with respect to other groups of microorganisms. In this way, both checkerboard and *in situ* hybridization could be used to study population distribution in this ecosystem.

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