MARINE BIOLOGICAL LABORATORY

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WOODS HOLE

MICROBIAL DIVERSITY IN ANOXIC MARINE SEDIMENTS

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INTRODUCTION

In anoxic sediments, the decomposition of organic matter to inorganic nutrients, CO_2 and CH_4, occurs through microbial metabolic reactions that produce increasingly less complex, dissolved, organic compounds from the sedimentary organic matter. The end products of one set of reactions are generally utilized as substrates by another set of organisms, leading eventually to the complete remineralization of the sedimentary organic matter. These processes appear to produce a limited number of substrates (*e.g.* acetate and H_2) which are then consumed by the appropriate terminal organisms (either sulfate reducers or methanogens). After oxygen is consumed by aerobic respiration, sulfate reduction becomes the dominant form of respiration. Methane generation and accumulation becomes dominant only after sulfate in sediment pore water is depleted. Of particular interest is the near absence of methane in the high sulfate zone in marine sediments, with methane concentrations increasing in the deeper, nearly sulfate-free sediments.

The aim of this study is to describe the microbial population in coastal anoxic marine sediments, with particular emphasis on sulfate reducing bacteria (SRB) and methanogens, (the latter being the most likely representatives of the *Archaea* domain to be found in anoxic cold marine sediments) using a combined microbiological/molecular approach. The combined application of molecular techniques, which do not require selective cultivation, with physiological/ecological approches in marine microbiology offers a means to facilitate the identification and quantitation of individual microbial species in naturally occurring microbial communities.

In situ hybridization with domain and group specific probes is used as a preliminary step to establish the presence of both *Bacteria* and *Archaea* in the untreated sediment samples. Selective enrichment cultivation of sulfate reducers and methanogens is combined in this study with 16S rRNA gene sequence analysis of sediment population. The combined approaches utilized in this study resulted in the description of different SRB that can be cultivated and in the identification of a number of widely diverse 16S rRNA gene sequences from *Bacteria* and *Archaea*. The comparative rRNA gene analysis of both cultured and uncultured bacterial types will place them in a phylogenetic framework, and will eventually provide information to develop a set of specific probes that could be used for future investigations of the sediment community structure.

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MATERIALS AND METHODS

Sampling procedures. A gravity core sample was collected in Buzzards Bay, Cape Cod, at a depth of 12m from the SSV *Corwith Cramer* (2 July 1995). As soon as the core was retrieved, the grey mud sediment was cut apart along its longitudinal axis and sub-samples were collected. The core was 75 cm long and a 3 cm thick black layer was clearly visible at a distance of about 10 cm from the top of the core. Large samples to be used as inocula for enrichment cultures were collected both from the black layer and from the bottom layer of the core and were stored in the dark. Small sub-samples from both the black and the bottom layer were quick frozen aboard the ship and kept at -20°C in the laboratory until they were processed.

Microscopy. Five ml of fixation buffer were added to 1g of sediment and the mix was vortexed at high setting. The mix was then put on ice for 2 min, the supernatant was discarded and the pellet was re-extracted with 2ml of fixation buffer. Cells were then fixed for 3-16h at 0-4°C. The fixation mix was centrifuged, washed twice with PBS, resuspended in 500µl of PBS plus 500µl of ethanol and stored a -20°C. Ten µl of a 100 fold dilution of cell suspension was applied to each well on the slide, allowed to dry for 20 min at 45°C and finally dehydrated for 3 min each time in 50%, 80% and 96% ethanol/H_2O. Forty µl of hybridization solution containing the fluorescent labeled probe were applied to each well and incubated overnight at the estimated hybridization temperature of the probe. Slides were then rinsed with SET solution (pre-warmed at the hybridization temperature), washed 3 times for 20 min each with SET, rinsed with dH_2O and finally dried. Ten µl of SET and 1µl of DAPI solution (1µg/ml) were added to each well and incubated at RT for 5-10 min, rinsed shortly with dH_2O and air dried.

Enrichment cultures. Enrichment cultures for SRB and methanogenic bacteria were carried out in liquid saltwater media, prepared according to the Course laboratory manual. Estimation of formed sulfide in SRB cultures was performed according the Schnell procedure.

Genomic DNA extraction. Genomic DNA was extracted from both the black and bottom layer of the the sediment samples according to the protocol developed by Sghir and Dore (unpublished). About 100 mg of sediment were thawed on ice, resuspended in TE (Tris HCl 10mM-EDTA 1mM) and enzymatic cellular lysis was used to maximize the recovery of high molecular weight DNA amenable to PCR. No physical treatment was used in order to prevent DNA shearing. Sediment samples were incubated with lysozyme, pronase, mutanolysin and RNAse at 37°C and then at 55°C after the addition of SDS and proteinase K. The polysaccharides and residual proteins were aggregated and extracted twice with an equal volume

of phenol, followed by a second double extraction with an equal volume of phenol-chloroform-isoamyl alcohol (50:49:1) and a third double extraction with an equal volume of chloroform-isoamyl alcohol (24:1) to remove the residual phenol. The genomic DNA was allowed to precipitate for 1 hour on ice after addition of 0.1 volume of 3M sodium acetate (pH 4.6) and 2 volumes of 100% cold ethanol. The DNA was then collected by centrifugation, washed in cold 70% ethanol, dried and resuspended in sterile distilled water.

Amplification of 16S rRNA gene sequences. The 16S rRNA gene sequences were selectively amplified from crude genomic DNA by PCR (Saiki et al., 1988) using primers designed to anneal to the conserved position in the 3' and 5' regions of 16S rRNA genes. The forward primer was 008F: 5'-AGAGTTTGATCCTGGCTCAG-3'; the reverse primer was 1517R: 5'-ACGGCTACCTTGTTACGACTT-3'. These oligonucleotides correspond to highly conserved or "universal" regions found in all known small-subunit ribosomal genes. The forward primer 344F: 5'-ACGGGGCGCAGCAGGCGCGA-3' was used together with primer 1517R to selectively amplify archaeal 16S rRNA genes. The reverse primer 385R: 5-CGGCGTCGCTGCGTCAGG-3' was used in combination with primer 008F to selectively amplify SRB 16S rRNA gene fragments. About 50 ng of template DNA were incubated in a thermal cycler in the presence of *Taq* DNA polymerase for 30 cycles under the following conditions: 92 degrees, 2 min; 48 degrees, 30 sec; 72 degrees, 30 sec. PCR products were purified and resuspended in sterile distilled water.

Construction of a 16S rRNA genes clone library. Amplified 16S rRNA gene fragments were cloned into the cloning site of pCR II plasmid (Invitrogen TA cloning kit) by sticky-end ligation. The resulting ligation products were used to transform competent *E. coli* INVαF' cells. Clones were screened for α-complementation and white colonies containing inserts were selected. Clones containing inserts of the appropriate size were identified by direct PCR screening followed by gel electrophoresis of the amplified insert.

16S rRNA genes RFLP analysis. Primary restriction of insert 16S rRNA gene fragments was performed by digesting the amplified 16S rRNA genes with Sau 3AI restriction endonuclease. The reaction products were visualized by electrophoresis on a 2.5% w/v agarose gel.

DNA sequencing and similarity analysis. rRNA genes from representatives of different categories of clones defined by RFLP were sequenced. Double stranded plasmid DNA templates for sequencing library clones were prepared and sequenced by standard dideoxynucleotide chain-termination methods (Sanger et al., 1977) using M13 forward fluorescent dye labeled

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primer. Sequence electrophoresis was performed on a Li-Core 4000 automated sequencing apparatus. The similarity analysis was performed using the program BLASTN, which compares a given sequence to a 16S rRNA sequence database and returns a list the most similar sequences found.

RESULTS

Microscopy. Subsamples of the core sediment (black layer) have been examined under the phase-contrast microscope as a preliminary approach to detect the precence of bacterial cells. No cells could be directly detected under the microscope due to the presence of particulate material in the sediment sample (Fig.1). DAPI stained bacterial cells could indeed be clearly detected in the sediment subsamples, often associated with the particulate material. Chains of cocci, sarcina-like associations, rods and vibrios were the most represented morphotypes (Fig.1). *In situ* hybridization on the same sample using a fluorescent-labeled 16S rRNA-based universal probe revealed only a minor fraction of the cells detected by DAPI staining (Data not shown). In addition to that, the few cells detected by *in situ* hybridization showed a very low level of fluorescence. Since DAPI binds to DNA while the rRNA-based probe hybridizes to the cellular 16S rRNA, this result could imply a low rRNA content in the bacterial cells. Since rRNA content is usually proportional to the growth rate, it seems likely that most of the bacterial cells detected in the subsample were not active. Actively growing cells in the enrichment for SRB at 18°C could be clearly revealed by DAPI staining and by *in-situ* hybridization using both a SRB specific fluorescent-labeled probe and a universal probe (Fig.2a).

Enrichment cultures. Enrichment cultures for both SRB and for methanogenic bacteria were carried out by inoculating subsamples of the black layer sediment and incubating them at three different temperatures: 18°C, 35°C and 55°C. No methanogenic bacteria were detected in any of the three conditions tested within the time schedule of the course. Enrichment cultures for SRB were carried out using either lactate, acetate or propionate as electron donors. Within four days from the inocula bacteria were actively growing in the two lactate-containing samples incubated at 35°C. No formation of sulfide could be detected in these enrichments (Tab. 1). Within four days from the inoculations, both bacterial growth and the formation of 4-10 mM H_2S could be detected in the enrichment carried out at 35°C in the precence of acetate as an electron donor. The cells, mainly vibrios and curved rods, appeared to be very motile. Bacteria were actively growing in the enrichment carried out at 55°C in the presence of acetate at day 5 from the inocula. The estimated amount of formed sulfide in the culture was about 20 mM. The culture appeared to be rather homogeneous under the microscope, the predominant morphotype being represented by spore-forming rods (Fig.2b). A 0.3 ml aliquot of this enrichment culture

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was tranferred in bottles containing fresh medium which were then incubated both at 55°C and at 70°C. Agar shake dilutions were made to isolate single colonies, which were then inoculated in liquid medium and incubated at 55°C. Examination of one of the single colonies under the microscope revealed the presence of a pure culture of the spore-forming rods. No growth was detected either in the 55°C and 70°C transfers or in the single colony inoculum within the time schedule of the course. Ten days after the primary inoculation both bacterial growth and production of sulfide were detected in the lactate containing enrichment incubated at 35°C. The predominant morphotype in this enrichment was represented by vibrio-like motile cells.

Enrichment	Growth	Estimated H_2S	Microscopy	In-situ hybrid
Lactate, 35°C/1	+	-	rods	nd
Lactate, 35°C/2	+	-	rods	nd
Acetate, 35°C	+	4-10 mM	Curved rods, cocci	-/+
Acetate, 55°C	+	~ 20 mM	Spore-forming rods	-/+
Lactate, 18°C	+	4-10 mM	Curved rods	++

Table 1: SRB enrichments.

Estimation of molecular diversity. Crude DNA was extracted both from the black laye and the bottom sediments and was used as a template to amplify 16S rRNA genes. Several dilutions of the crude DNA extract were used in the primary amplification to dilute eventual inhibitory substances present in the sediment. Figure 3 clearly shows that the optimal dilution for PCR amplification was 10^{-3}, while higher concentrations of template DNA did not result in a detectable PCR product (Fig.3). Three sets of primers were used: universal, archaeal domain-specific and SRB group-specific, which generated respectively a 1509, 1173 and 377 bp PCR product. After cloning in pCR II plasmid vector, *E. coli* INVαF' cells were transformed with the ligation products. A total of 60 colonies were chosen after they tested positive for α-complementation of β-galactosidase, and 59 clones contained an insertion detectable by PCR. All but two of the clones contained an insert of the correct size (Fig.4). A RFLP analysis was performed on 50 of the amplification products of the correct size using Sau3AI restriction endonuclease (Fig.5). Restriction of the 50 intact 16S rRNA gene fragments with Sau 3AI

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indicated that many diffrerent patterns were obtained. Seventeen clones could be grouped in 7 different RFLP patterns, while the remaining 41 clones appeared to be unique.

Sequence similarity analysis. Ten unique clones representative each of the three primer sets (Universal, archaeal and SRB) were selected on the basis of their different Sau3AI RFLP and were sequenced using a Li-core 4000 automated sequencer. The obtained sequences ranged from about 240 to 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 program used in this analysis was BLASTN, which compares query sequences to the database sequences and returns a list of the most significant sequence similarities found. Four universal primer-amplified rRNA genes were sequenced and searched by BLAST: two of these sequences returned a list of different SRB16S rRNA genes as the most similar sequences found; one of them was found to be similar to two marine *Spirochaeta* 16S rRNA genes. The BLAST search for the fourth sequence returned a list of thermophilic Bacteria (mainly *Thermus* sp.), the most similar ones referring to microorganisms isolated from a Yellowstone Park freshwater hot spring. Three archaeal primer-amplified rRNA genes were sequenced. The similarity search for all of them returned a striking result: the most similar sequences found for each query belonged to the 16S rRNA genes of Crenarchaeotal sp.clones (pJP89, pJP33 and pJP41, see Fig.6) isolated from a Yellowstone hot spring environment (Barns et al. 1994). A number of other 16S rRNA genes from hyperthermophilic *Archaea*, both from marine and freshwater geothermal environments, followed in the high-scoring producing sequence list.

DISCUSSION

Direct *in situ* hybridization on fixed sediment subsamples from the black layer with universal probes, archaeal domain-specific probes and SRB group-specific probes failed to reveal the presence of bacterial cells. In some cases two different probes, either universal or archaeal, have been used in combination in an effort to obtain a more powerful signal. Since DAPI staining of the same samples revealed, on the other hand, a considerable amount of morphologically diverse microorganisms, most of them associated with the sediment particulate material, we can conclude that in this case the sensitivity of the fluorescent-labeled probe essay is too low to reveal the presence of the cells. This is probably due to the low amount of rRNA present in the cells. On the base of this results it is possible to speculate about the relative low activity of the bacterial cells which could be detected with the DAPI staining procedure while could not be revealed by fluorescent labeling rRNA-based probes.

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Enrichment cultures for SRB have been carried out using different electron donors and different incubation temperatures to select for diverse microorganisms. Different enrichments have indeed been obtained (Fig. 2a and 2b). The isolation efforts have been focussed on the 55°C enrichments, since I found intriguing the possibility to enrich for a moderate thermophilic SRB from cold marine sediments. An attempt for clearly thermophilic growth has been done incubating a transfer from the primary 55°C enrichment at 70°C, but no growth has been detected in this sample within the time schedule of the course. Due to time costraints it is not been possible to grow isolated colonies of the spore-forming rod morphotype in pure liquid cultures. Since all spore-forming SRB known to date are classified in the genus *Desulfotomaculum*, it is likely that the 55°C spore-forming SRB which have been isolated belongs to the same genus . Moreover the *Desulfotomaculum* genus contains a number of moderately thermophilic species with temperature optima of 54°C to 65°C (*e.g. D. geothermicum* and *D. nigrificans*). Since only one isolation of a still unnamed marine *Desulfotomaculum* strain has been reported to date (Keith et al., 1982), while most of the known species are from freshwater habitats, it would be interesting to furtherly characterize the 55°C growing putative *Desulfotomaculum* strain. It would certainly be very interesting to obtain the 16S rRNA gene sequence from a pure culture of this isolate, and to compare it to the collection of sequences retrieved directly from the sediment sample in order to understand wether it is represented in the collection. Furthermore, a genus or even a species-specific probe could be derived from the sequence of the pure isolate in order to probe the original sample and quantify the relative abundance of *Desulfotomaculum* species in the sediment with respect to other groups of microorganisms, although a procedure to enhance the sensitivity of the direct fluorescent labeling of cells in the sediments should be worked out.

The RFLP analysis showed a remarkable molecular diversity among the 16S rRNA genes retrieved from the sediment, both in the *Bacteria* and in the *Archaea* domain. The three Archaeal 16S rRNA gene sequences we cloned from the sediment subsample appeared to have a different Sau3AI RFLP pattern. When sequenced, the three clones showed to be indeed different, although the most striking observation was that the most similar sequences found in the database, for all the three query sequences, were a cluster of sequences from both yet uncultivated *Crenoarchaeota* and isolated and well characterized hyperthermophilic *Archaea.* (Fig.6). These results are however preliminary and should be confirmed by sequencing the complete 16S rRNA genes, although the similarity score obtained in the BLAST search was very high for at least two out of three sequences. An alignment of each of the complete archaeal sequences obtained should place them in the right phylogenetic framework and indicate the closest relative to each of our clones. Although this is probably the first report of putative crenoarchaeotal sequences retrieved from cold anoxic marine sediments, there have been reports

about the retrieval of both crenoarchaeotal and euryarchaeotal sequences in oxygenated water off the North American coast and in Antarctica (DeLong, 1992; DeLong et al., 1994). In marine cold habitats, sediment dwelling *Archaea* usually belong to the methanogens group. Other common archaeal marine habitats are generally limited to shallow or deep-sea hydrothermal vents (methanogens and extreme thermophiles) and to highly saline land-locked seas (halophiles). The natural question that arises from the present study is: why do we find microorganisms related to the thermophiles group in a cold habitat, and how do such microorganisms make a living there?

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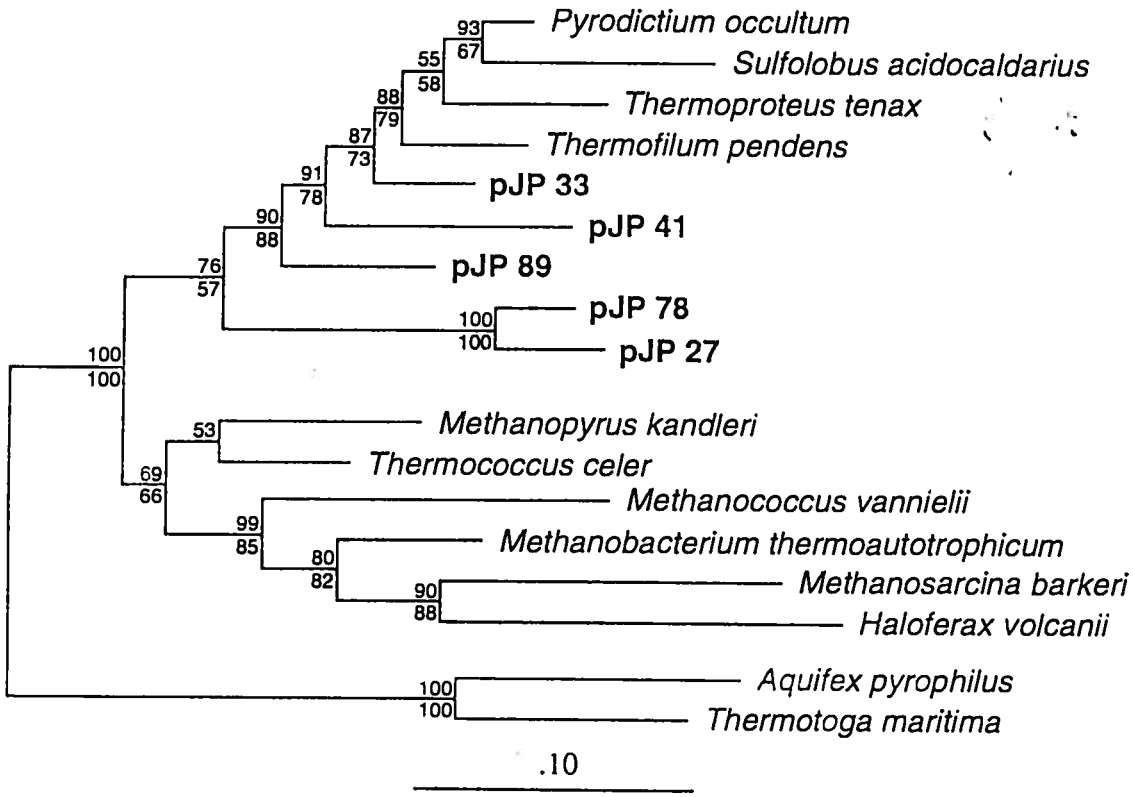

Fig 6: Archaeal phylogenetic tree (Barns et al., 1994)

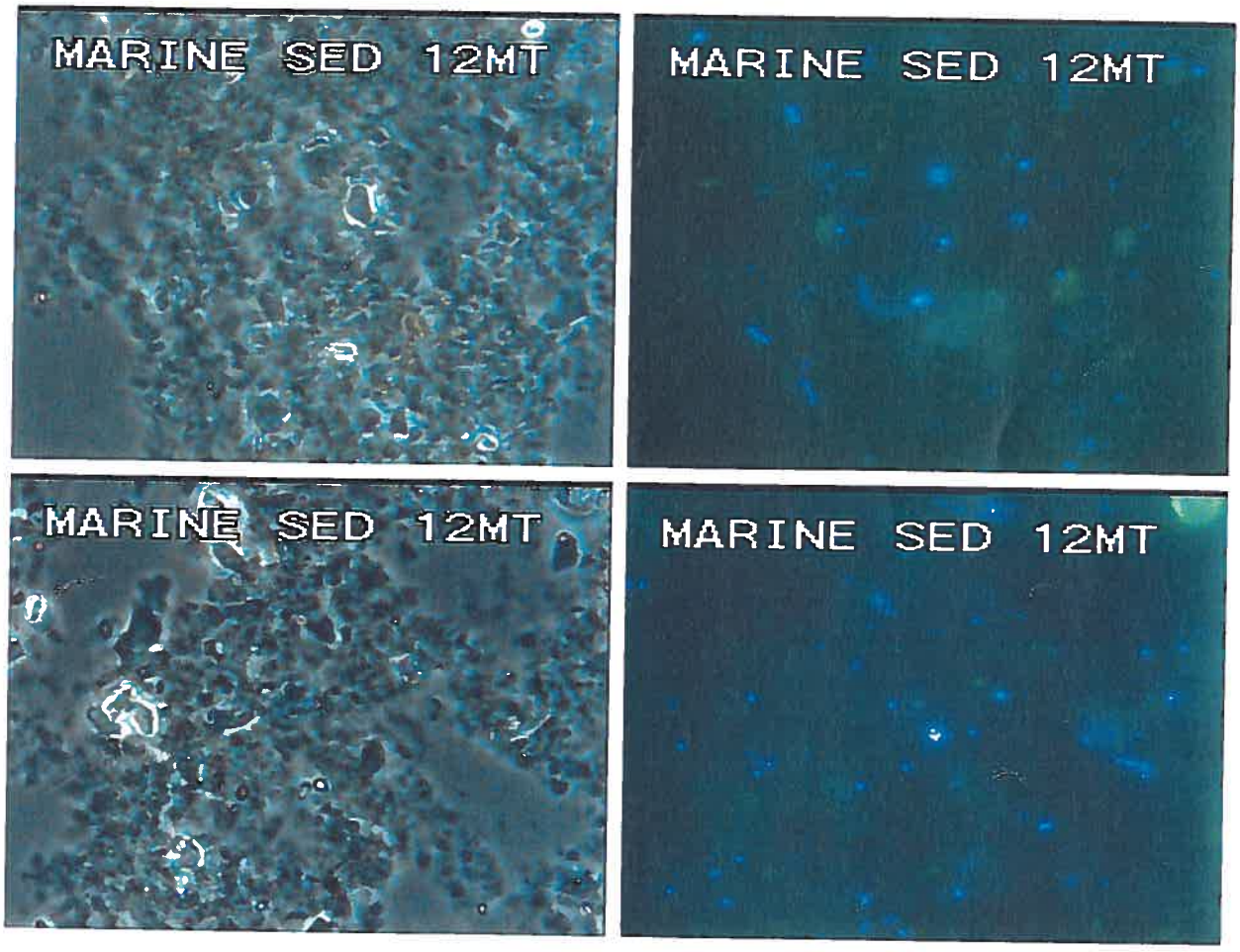

Fig 1: Phase contrast microscopy (left) and DAPI staining (right) of marine sediments

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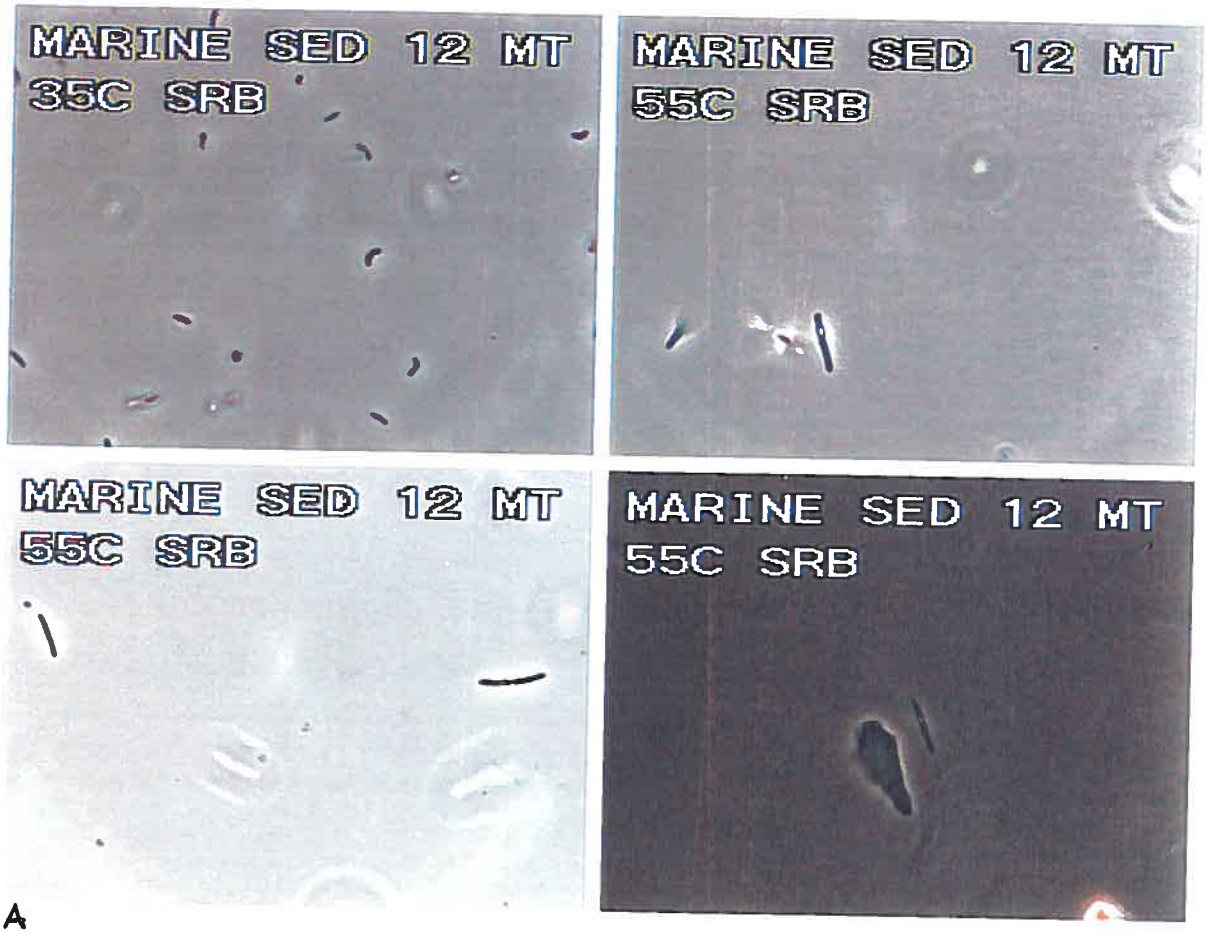
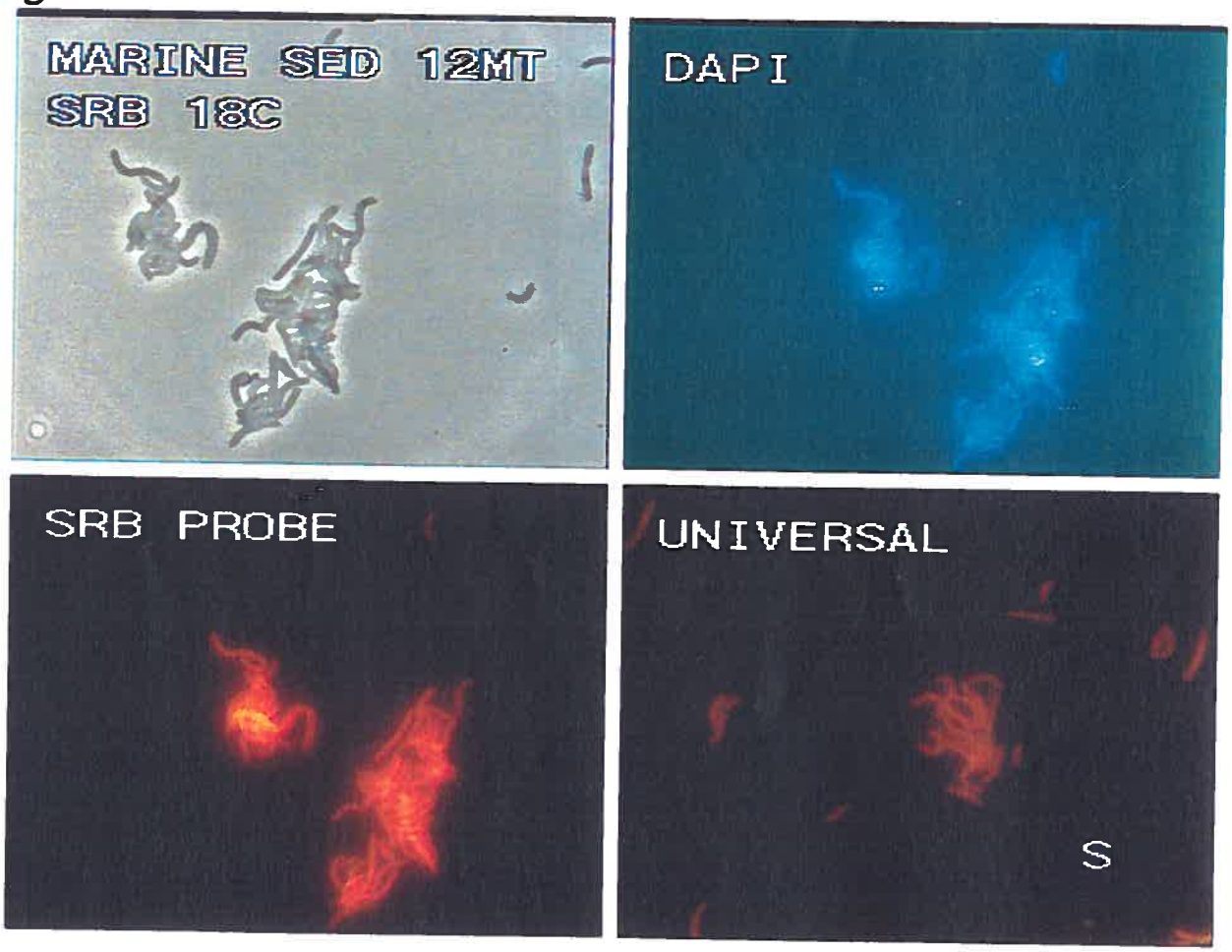


A

Fig 2: SRB enrichments

B



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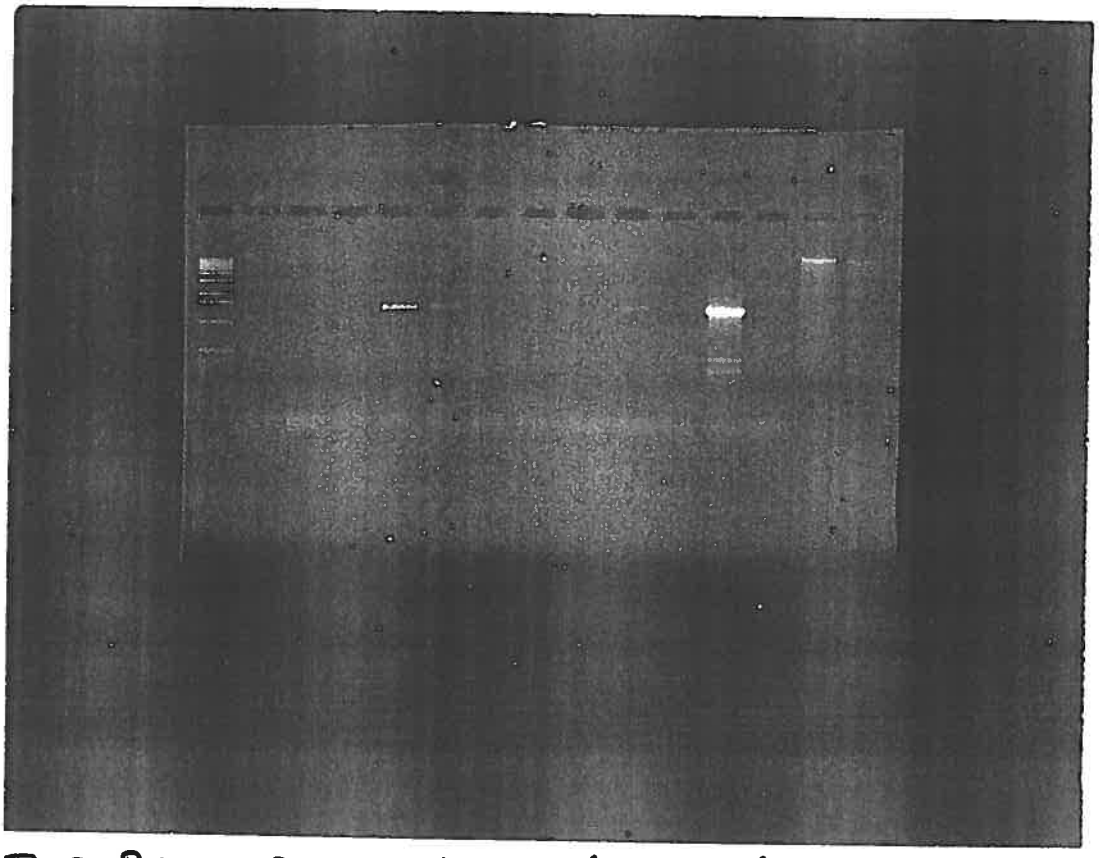

Fig 3: Primary PCR amplification of 16S rRNA from crude DNA extracts
lane 1: 1000 bp ladder
lane 2 to 6: 16S rRNA genes, black sediment. ud to 10^{-4}
lane 7 to 11: 16S rRNA genes, bottom sediment. ud to 10^{-4}
lane 12: Positive control
lane 13: Negative control
lane 14: Genomic DNA, black layer
lane 15: Genomic DNA, bottom layer

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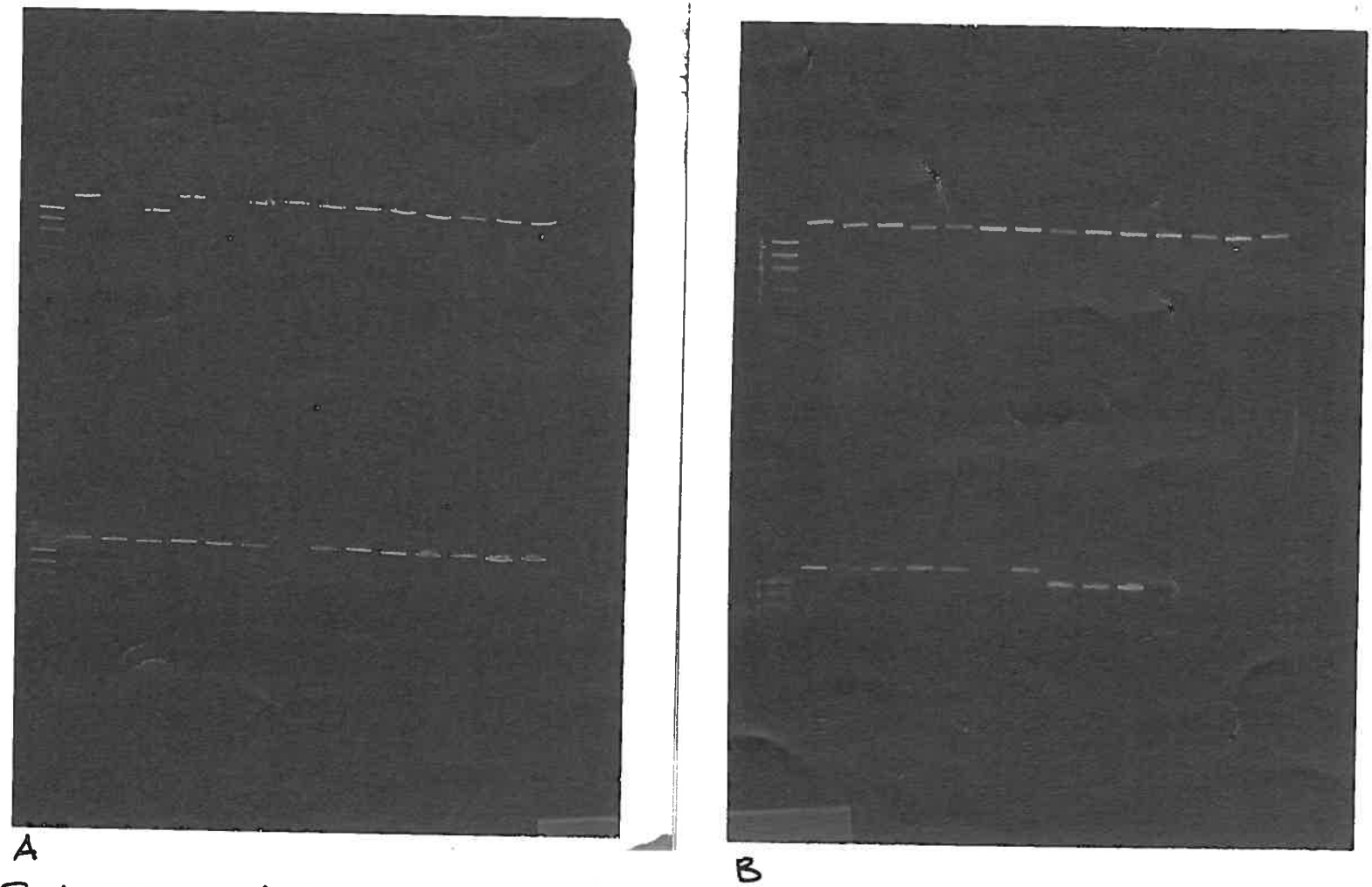
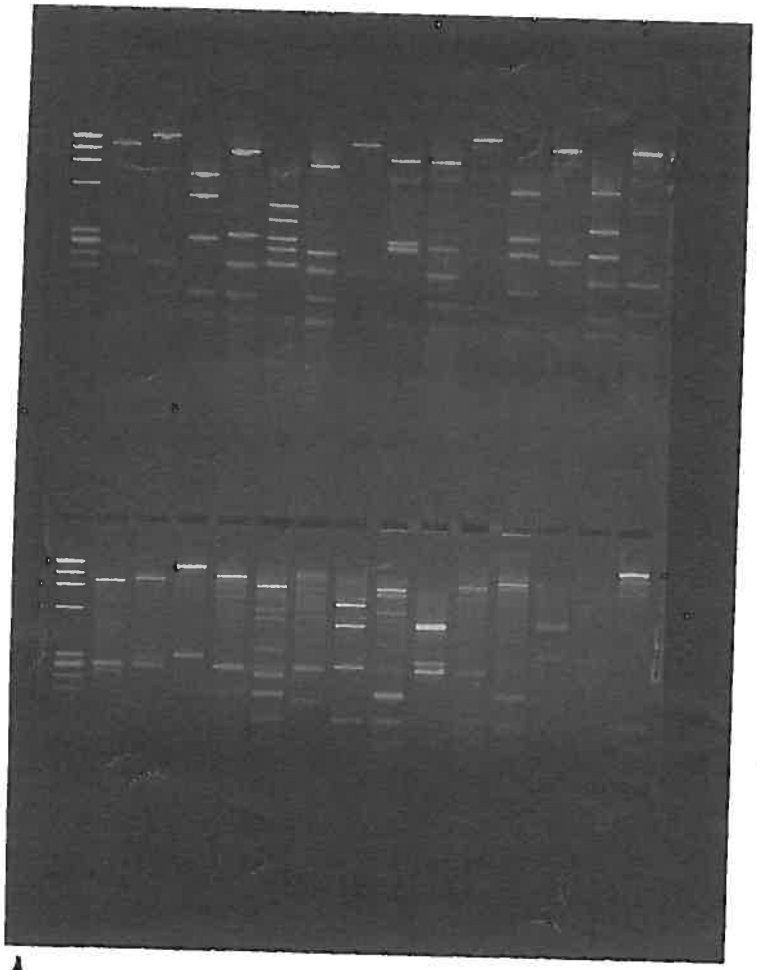


A B

Fig 4: PCR amplified 16S rRNA gene fragments
A: Universal primers
B, upper gel: Universal primers
B, lower gel: lanes 2 to 5, Universal primers - lanes 8 to 11: Archaeal primers - lanes 12 to 15: SRB primers
Marker: ϕX174 HaeIII



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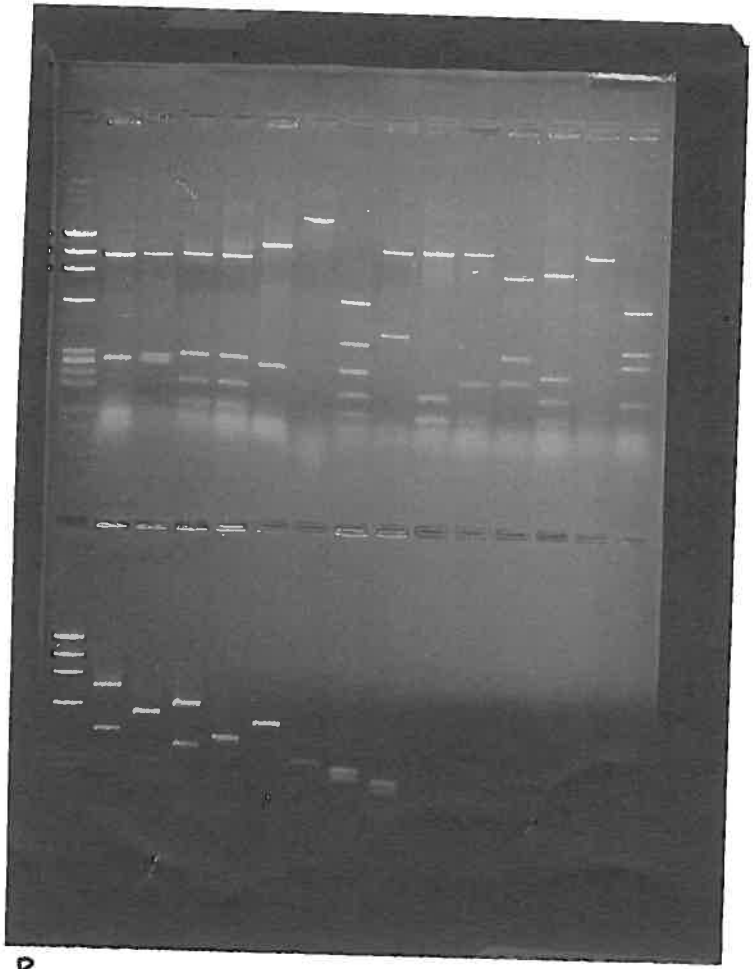

B

Fig 5: Sau3AI RFLP
A: Universal primers
B: lane 2 to 4 : Archaeal primers
lane 5 to 9 : SRB primers

methanogens could have grown. These were not observed microscopically or tested in any way, therefore one cannot definitively say that methanogens grew in these enrichments.

In conclusion, the massive presence of iron in parts of Sippewissett salt marsh suggest that it is possible that iron reducers can account for a great deal of carbon mineralization. Results obtained so far from the primary enrichments are very positive in determining the presence of iron reducers at Sippewissett salt marsh. Much more work needs to be done to definitively declare that iron reducers are in the salt marsh. Additionally, pure cultures need to be obtained first before metabolic versatility can be addressed.

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