# In Search of Archaea among the Mat Layers of the Great Sippewissett Salt Marsh

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## Abstract

Microbial mats are stratified layers of diverse microbial consortia that generally consist of a surface layer dominated by cyanobacteria with lower layers composed of purple anoxygenic phototrophic and sulfate reducing bacteria, among others. While the bacterial component has been the focus of most studies on microbial mat communities, the presence and role of archaea remains largely unstudied. Recent phylogenetic analyses have revealed that archaea are ubiquitous, and thus, there is no *a priori* reason why archaea should not be present in the complex microbial assemblages of the mats. Our group took a polyphasic approach to determine if archaea are present in the mat layers of the Great Sippewissett Salt Marsh that included: i) enrichment and isolation of methanogenic archaea, ii) $F_{420}$ autofluorescence (characteristic of methanogenic archaea) of cells grown in enrichment cultures, iii) *in situ* hybridization of archaea-specific DNA probes to cells grown in enrichment cultures, and iv) sequence analysis of archael 16S rDNA that was PCR amplified from both enrichment cultures and directly from the mat layers. After one week of incubation at 22°C, bubble formation from in tact mat layers added to enrichment media was our first indication that methanogenesis was taking place. Cells from these enrichments autofluoresced when viewed by epi-fluorescence microscopy at 420 nm and their DNA hybridized to archaea-specific DNA probes. The morphology of these cells (packets of coccoid cells) and their ability to use methanol as a sole substrate for methanogenesis suggested that they were members of the genus *Methanosarcina*. Sequence analysis of the single 16S rDNA RFLP type amplified from these enrichments suggested that an archeon most closely related to *Methanogenium* was present. Archaeal 16S rDNA was also amplified directly from the mat layers. At least 16 different RFLP patterns were detected, suggesting that a diverse archael consortium may be present. Sequence analysis of these cloned 16S rDNA genes is in progress and will i) allow us to infer phylogeny of these archaea, ii) allow us to infer the metabolic capabilities of these archaea based on their phylogenetic grouping, and iii) will serve as a source of archael DNA for probe design to study their ecology within the mats.

## Introduction

Microbial mats are stratified layers of complex microbial associations. The dominant primary producers of mats are the oxygenic photosynthesizing cyanobacteria. Common genera of cyanobacteria found in mats are *Nostoc*, *Anabaena*, *Oscillatoria*, and *Spirulina*. Below the surface mat layer(s) are diverse assemblages of bacteria that include members of the purple anoxygenic photosynthesizing bacteria and sulfate reducing bacteria; the latter can catabolize photosynthate in the presence of sulfate (derived from seawater or other sources) to produce sulfide. Purple sulfur bacteria are in turn able to utilize the sulfide for their metabolic needs. Cyanobacterial filaments produce extracellular mucopolysaccharide sheaths (from photosynthate) that give overall cohesion to the mat and protect it from disintegration and drying during low tide cycles. Studies of these and other complex associations within the mat layers have focused largely on understanding the bacterial component of the mats, with little or no attention given to the presence and role of archaea. Our group took a polyphasic approach to determine if archaea are present in the mat layers that included: i) enrichment and pure culture isolation, ii) microscopy, iii) *in situ* hybridization using archaea-specific DNA probes, and iv) sequence analysis of 16S rDNA. We emphasized the identification of methanogenic archaea by enrichment and microscopy because: i) they are responsible for $CH_4$ production in a variety of anaerobic marine and freshwater habitats, yet their presence in microbial mat layers, to our knowledge, is not documented, ii) they are relatively easy to detect due to their metabolic activity ($CH_4$ production), and iii) they possess a unique $F_{420}$ autofluorescence under epifluorescence microscopy. *In situ* hybridization using archael DNA probes and purification and sequencing of 16S rDNA from enrichment culture and mat samples were performed to provide a more comprehensive list of archaea that are present. The specific goals of this project were to: i) detect the presence of methanogenic archaea through enrichment and subsequent isolation, ii) use epifluorescence microscopy to detect $F_{420}$ autofluorescence and fluorescence due to hybridization to labeled archael probes, and iii) extract, PCR amplify, clone, and sequence archaeal 16S rDNA from both enrichment cultures and directly from mat layers.

## Materials and Methods

### Sampling and Storage of Mat Layers

Mat layer samples from the Great Sippewissett Salt Marsh were collected using a core sampler and either brought back to the lab within 3 h of collection or were inoculated directly into enrichment cultures. Unused mats were stored at 4°C for up to two weeks in case they were needed for further analyses. Individual mat layers (green, pink, black, and gray) were separated using a razor blade.

### Enrichment for Methanogenic Archaea

Mat layer samples were directly inoculated into anaerobic Basic Saltwater Medium th t was reduced by $Na_2S$ and supplemented with 10mM methanol (according to the recipe of B. Schink). Enrichment cultures were incubated at 22°C in the dark for up to 2 weeks. Due to a lack of time, agar shakes to isolate methanogens were not performed.

### Genomic DNA Extraction

Genomic DNA from 1 g of each mat layer and 0.5 g of sediment from a single enrichment culture containing all mat layers was extracted according to the procedure provided in the course handout. The only modification was that following the extraction procedure, DNA was purified using the Wizard DNA Purification Kit (Promega Corp.) according to manufacturer's specifications. Concentration of extracted DNA was estimated using the DNA Dipstick Kit (Invitrogen Corp.).

## PCR Amplification

To determine if the purified DNA contained any inhibitors of the PCR reaction, purified DNA was mixed with universal forward (5′ AGAGTTGATYMTGGC 3′) and reverse (5′GYTACCTTGTTACGACTT 3′) 16S rDNA primers and these PCR reactions were performed (according to the methods outlined in the course handout) prior to running PCR reactions using the more specific archael primers. To amplify archael 16S rDNA, two forward archael primers were used separately with the universal reverse primer. The forward archael primers were: AF1 (5′ TCCGGTDGATCCTGCCRG 3′) and AF21 (5′ TCCGGTTGATCCYGCCGGA 3′). PCR reactions containing 0.025 to 0.05 ng of purified DNA, 2.0 mM $MgCl_2$ and 0.5 µl (per 50 µl reaction) of AmpliTaq Gold DNA polymerase were cycled under the following conditions: 94°C, 5 min. (hot start), followed by 35 cycles of 94°C for 2 min, 63°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 10 min.

## Cloning and RFLP Analysis of PCR Products

PCR products were cloned into the PCRII plasmid (Invitrogen Corp.) according to manufacturer's specifications. Colonies of potential transformants were individually spotted onto an LB-ampicillin (100 µg/ml) plate (for storage following their growth) and then immediately added to separate PCR reactions which were mixed with TOPO forward and reverse primers (TOPO TA Cloning Kit, Invitrogen Corp) in order to amplify (and thus detect) the presence of the insert. PCR products from these reactions

## Cloning and RFLP Analysis of PCR Products cont.

were then mixed with *Hin*PI and *Msp*I (according to the course handout) and the resulting restriction patterns were analyzed by agarose gel electrophoresis. PCR products yielding unique restriction patterns were either sent out immediately for sequencing or frozen for future analyses; those PCR products sent out for sequencing were submitted to the BLAST database.

## *In Situ* Hybridization and Microscopic Analysis

Cells from methanogen enrichment cultures were concentrated by centrifugation and either viewed by epifluorescence microscopy for $F_{420}$ autofluorescence or were prepared for Fluorescent *In-Situ* Hybridization using the archael DNA probe ARCH (5′ GTGCTCCCCCGCCAATTCCT 3′) and DAPI counterstain according to the course handout prepared by S. Dawson.

## Results

### Evidence for Methanogenesis in Mat Layer Enrichments

After 1 week of incubation, bubble formation suggested that methanogenesis was taking place in the enrichment cultures. Formation of bubbles (presumably methane) subsequently increased during the second week of incubation.

### Extraction, Purification, PCR Amplification, Cloning, and RFLP Analysis of Archael 16S rDNA from Three of Four Mat layers and One Enrichment Culture

DNA isolated from both enrichment culture and directly from the four mat layers was initially brown in color suggesting that humics and other contaminants were present in the samples. Such DNA, when used in PCR reaction, would not amplify without further purification by passage through Wizard DNA Kit columns. In general, dilutions of up to 1:100 of the extracted DNA were also necessary to achieve optimal amplification. The addition of universal forward and reverse primers to PCR reactions yielded products of the appropriate size (1.5 kb) from all mat layers, however, we were initially unsuccessful at amplifying 16S rDNA using archael primers with all other conditions the same as those used for amplification with the universal primers. After repeated optimization steps using the Invitrogen PCR Optimizer Kit (according to manufacturer's specifications), an optimized scheme was devised for amplifying archael DNA from at least one layer (using Buffer B containing 2.0 mM $MgCl_2$, (pH 8.5) of the optimizer kit, 63°C primer annealing, and various dilutions of DNA of up to 1:100; data not shown). RFLP analysis of these PCR products revealed that at least 3 different restriction patterns were present. Interestingly, no amplification of any other layer was achieved using these optimized conditions. However, when Amplitaq Gold Polymerase (Perkin-Elmer Corp.) was used in place of Taq Beads, combined with the PCR protocol from the course handout,

**Extraction, Purification, PCR Amplification, Cloning, and RFLP Analysis of Archael 16S rDNA from Three of Four Mat layers and One Enrichment Culture cont.**

we were able to amplify archael DNA from both the gray and black mat layers (Fig. 1) as well as from enrichment culture, but not from the pink or green layers (data not shown). Subsequently, an aliquot of pink layer DNA was analyzed by agarose gel electrophoresis and appeared to be degraded. From two separate plates containing over 100 potential 16S rDNA insert-containing clones from both gray and black layer DNA samples, 20 were selected (10 from cloning reactions from each layer) for RFLP analysis. At least 14 different restriction patterns were represented in these samples (5 from the gray layer and 9 from the black layer; Fig. 2). A single RFLP pattern was achieved from the 16S rDNA amplified from enrichment culture and its sequence was most closely related to the archael genus *Methanogenium* (Fig. 3).

**$F_{420}$ Autofluorescence and *In Situ* Hybridization Revealed the Presence of Archaea in Enrichment Cultures.**

$F_{420}$ autofluorescence was detected in concentrated samples of methanogen enrichment cultures (Fig. 4), but was not detected in any mat layer directly. However, due to the large number of microbes in a typical mat layer sample (Fig. 6) the lesser abundant methanogens can easily be missed. The cells appeared to be coccoid in shape and arranged as packets of cells (Figs. 5a-b). Similarly, these cells hybridized to archaea-specific DNA probes (Fig. 5b). Their morphology and ability to utilize methanol as a sole substrate for methanogenesis suggests that they are members of the genus *Methanosarcina*. Interestingly, DNA of a filamentous bacterium (Fig. 7b) also reacted with the archaeal probe supporting the possibility that a diverse archael population may occur in the mat layers.

## Discussion

The results of our research provide evidence for the occurrence of a potentially diverse population of archaea within the mat layers of the Great Sippewissett Salt Marsh. 16S rDNA RFLP patterns suggest that at least 17 unique phylogenetic types of archaea are present in three of the mat layers (pink, black, and gray). Sequencing of these 16S rDNAs will be required in order to more accurately infer their phylogenies. Based on phenotypic data (metabolic and morphological) there is evidence to support that *Methanosarcina* may be present in the enrichment cultures, whereas phylogenetic analysis of the sole 16S rDNA clone suggests that the predominant archeon within the enrichments is most closely related to *Methanogenium.* Taken together, these data provide strong evidence to support that archaea are present in the mat layers. In the future, in order to isolate these methanogens and more accurately identify them, cultures should be started earlier. Interestingly, the potential archeon pictured in Fig 7b appears to be morphologically identical to an archeon from sulfide-rich sediment from the great Sippewissett Salt Marsh detected by S. Dawson in 1997. In conclusion, we have provided data fulfilling each of our goals as listed in the introduction and expect that this work will: i) provide the first additions to what should be a growing list of archaeal 16S rDNA sequences from the mat layers, ii) facilitate more detailed future studies on the phylogeny (and thus, metabolic capabilities) of archaea in the mat layers, and iii) facilitate probe design for ecological studies of these and other archaea.

## Figure Legends

**Fig. 1. PCR amplification of 16S rDNA using archael forward primer AF1 and universal reverse primer. Lanes 2-11: Black layer (top gel); Lanes 12-14 Gray Layer (top gel); Lanes 2-7: Gray layer (bottom gel)**

**Fig. 2. RFLP patterns of the PCR products from Fig. 1. First 10 of the "Pat" lanes: Black Layer; Last 10 of "Pat" lanes: Gray layer**

**Fig.3. Phylogenetic tree constructed using sequence data from the single 16S rDNA clone from enrichment culture**

**Fig. 4. $F_{420}$ autofluorescence of cells from enrichment culture**

**Fig. 5a. DAPI-stained cells from enrichment culture**

**Fig. 5b. Hybridization of DNA from cells in enrichment culture to an archaea-specific DNA probe**

**Fig. 6. An example of the morphological diversity of cells within the pink mat layer**

**Fig. 7a. DAPI-stained filamentous prokaryote from enrichment culture**

**Fig. 7b. Hybridization of DNA from the filamentous bacterium in 7a to an archaea-specific DNA probe**

**Appendix I Checkerboard hybridization on DIG-labeled PCR products from four layers of the microbial mat**

Fig 1

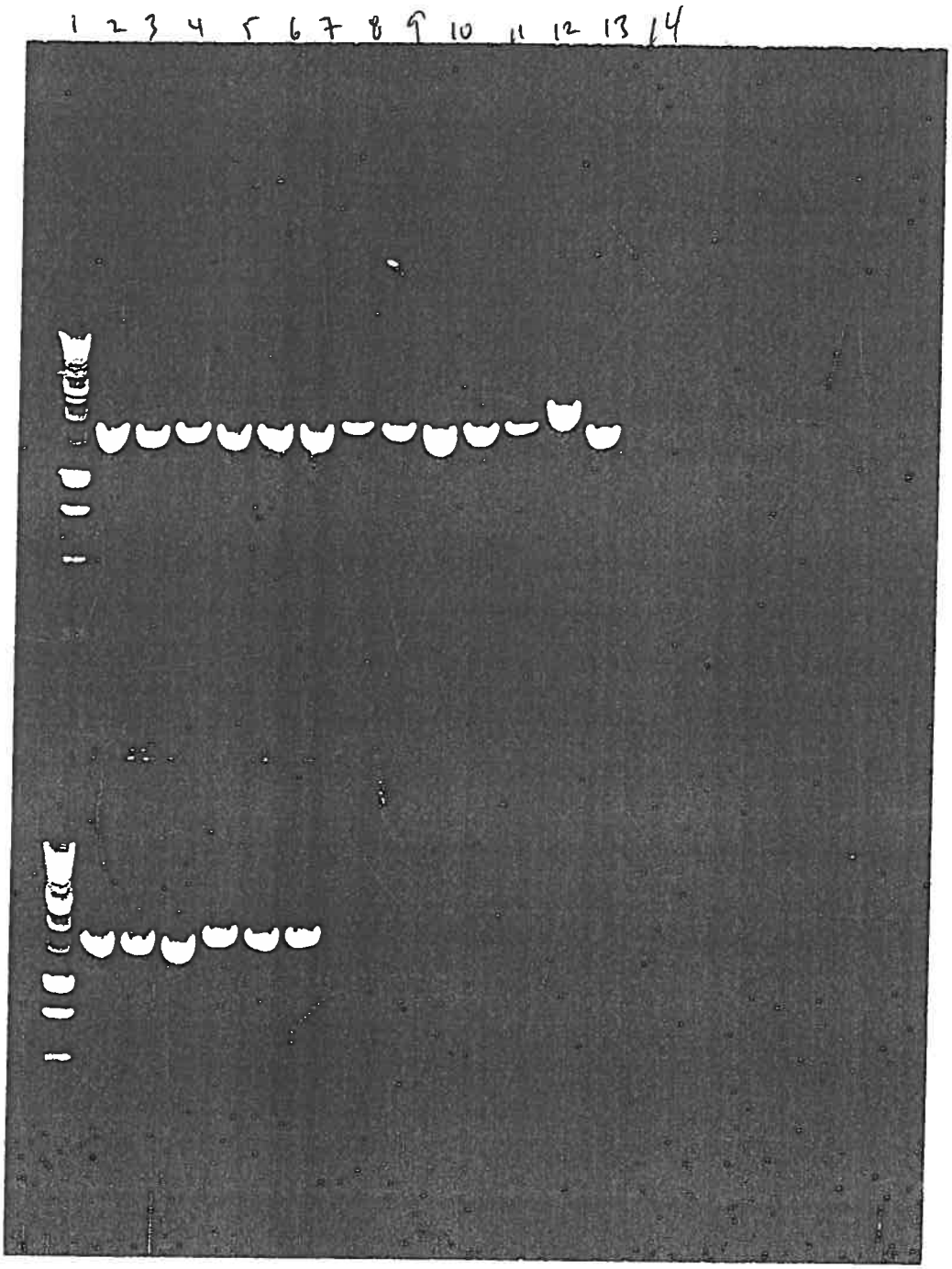
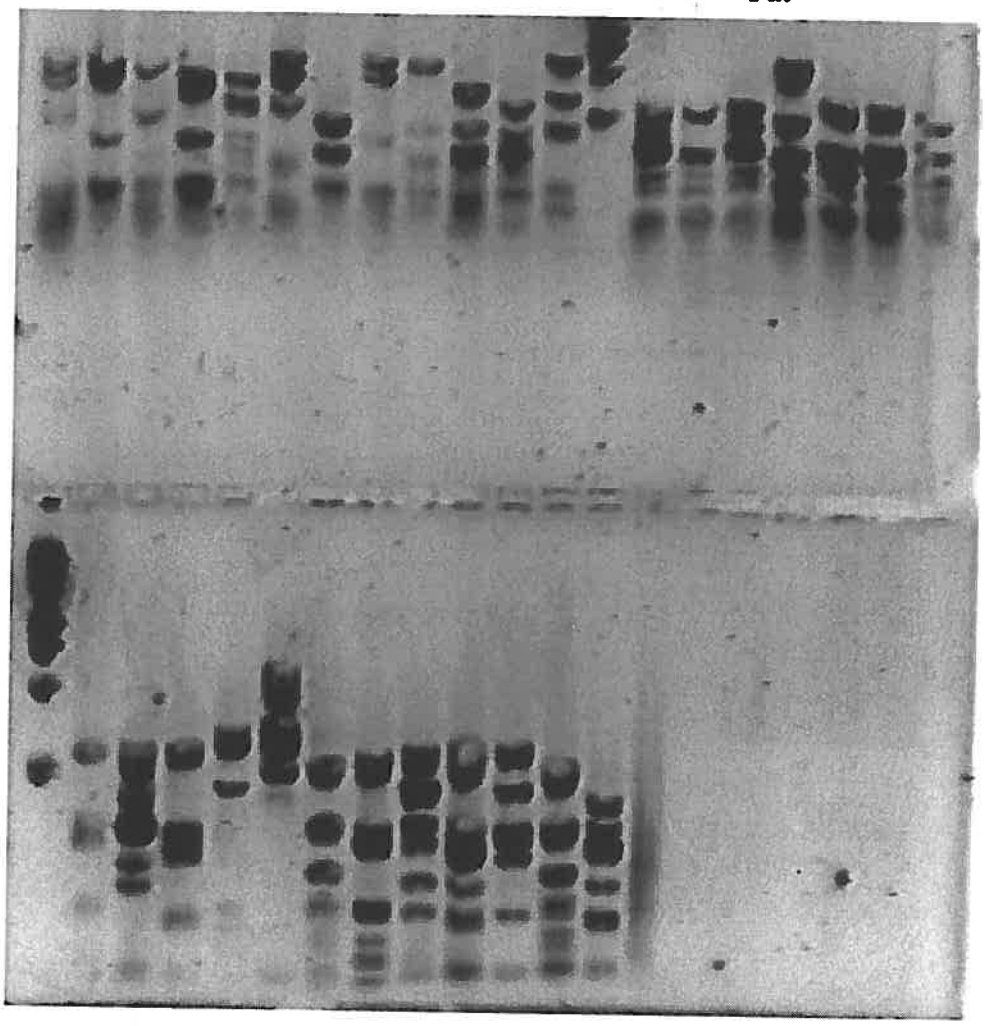


Fig 2

# RFLP Patterns

---------- Chris ---------- 1 KB --------- Pat ----------



1 KB---------- Pat ----------

For seq. - 1, 3-5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19

Fig 3

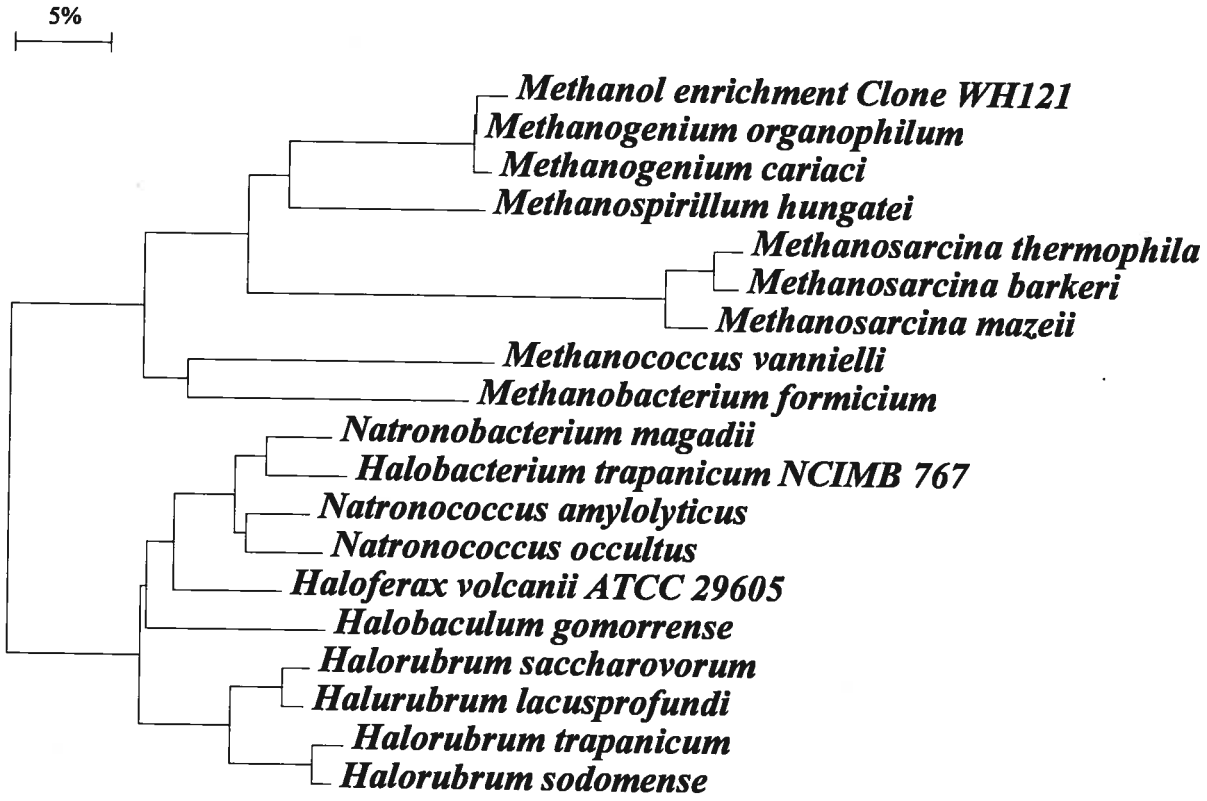

5%
Methanol enrichment Clone WH121
Methanogenium organophilum
Methanogenium cariaci
Methanospirillum hungatei
Methanosarcina thermophila
Methanosarcina barkeri
Methanosarcina mazeii
Methanococcus vannielli
Methanobacterium formicium
Natronobacterium magadii
Halobacterium trapanicum NCIMB 767
Natronococcus amylolyticus
Natronococcus occultus
Haloferax volcanii ATCC 29605
Halobaculum gomorrense
Halorubrum saccharovorum
Halurubrum lacusprofundi
Halorubrum trapanicum
Halorubrum sodomense

Fig. 4

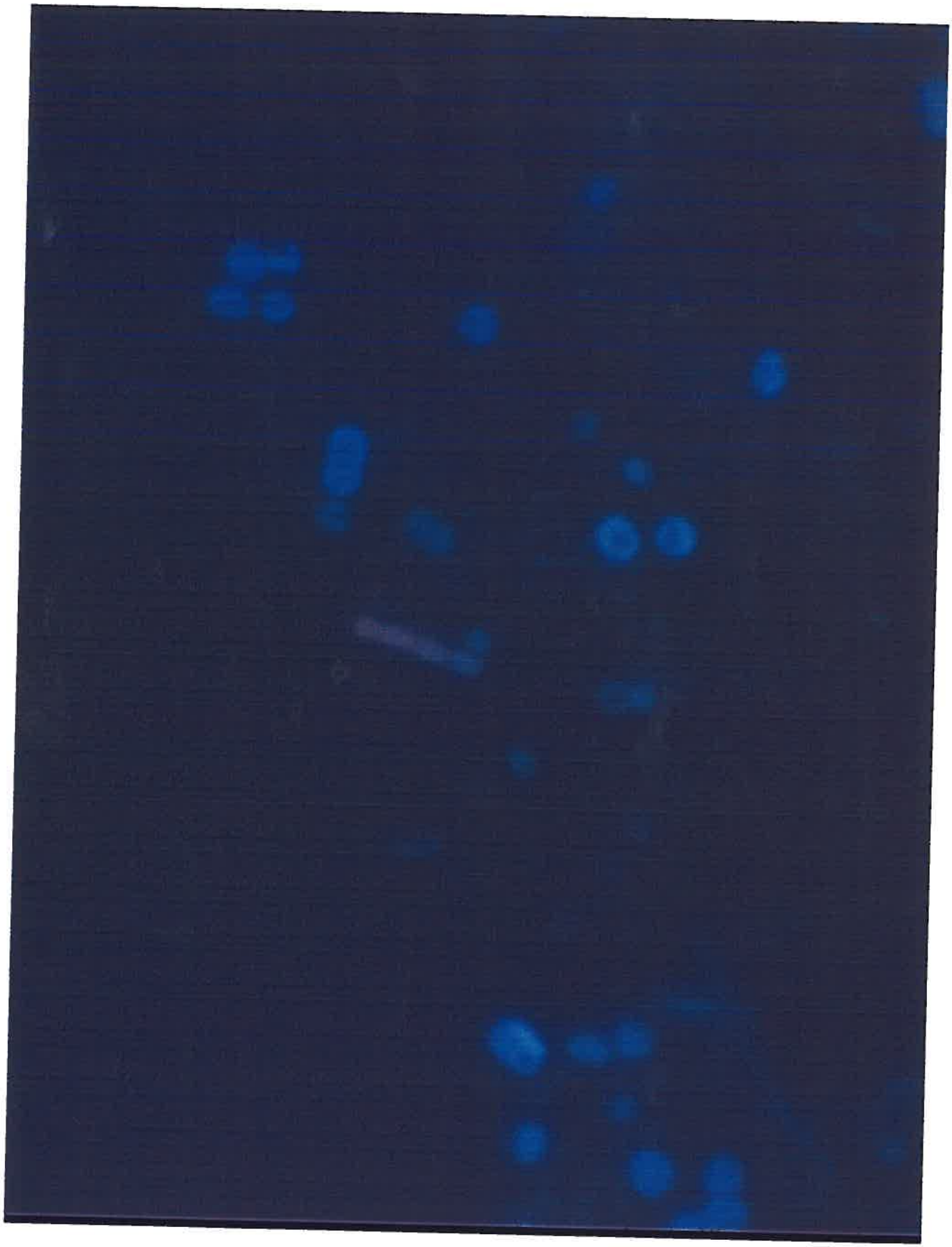


Fig 5a

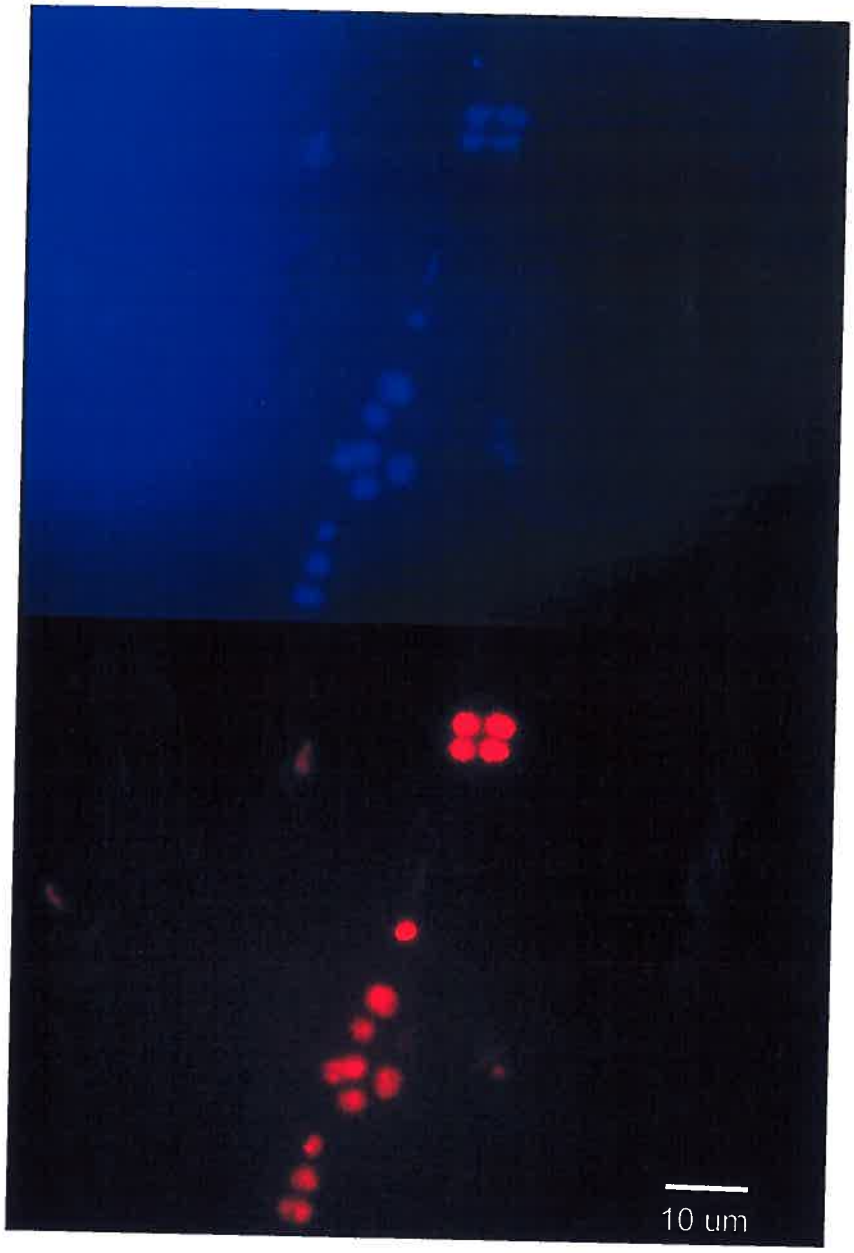



Fig 5b

Fig 6

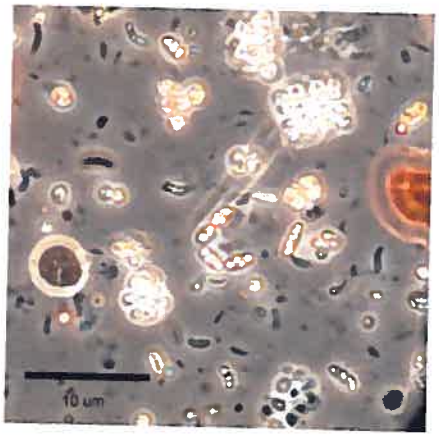
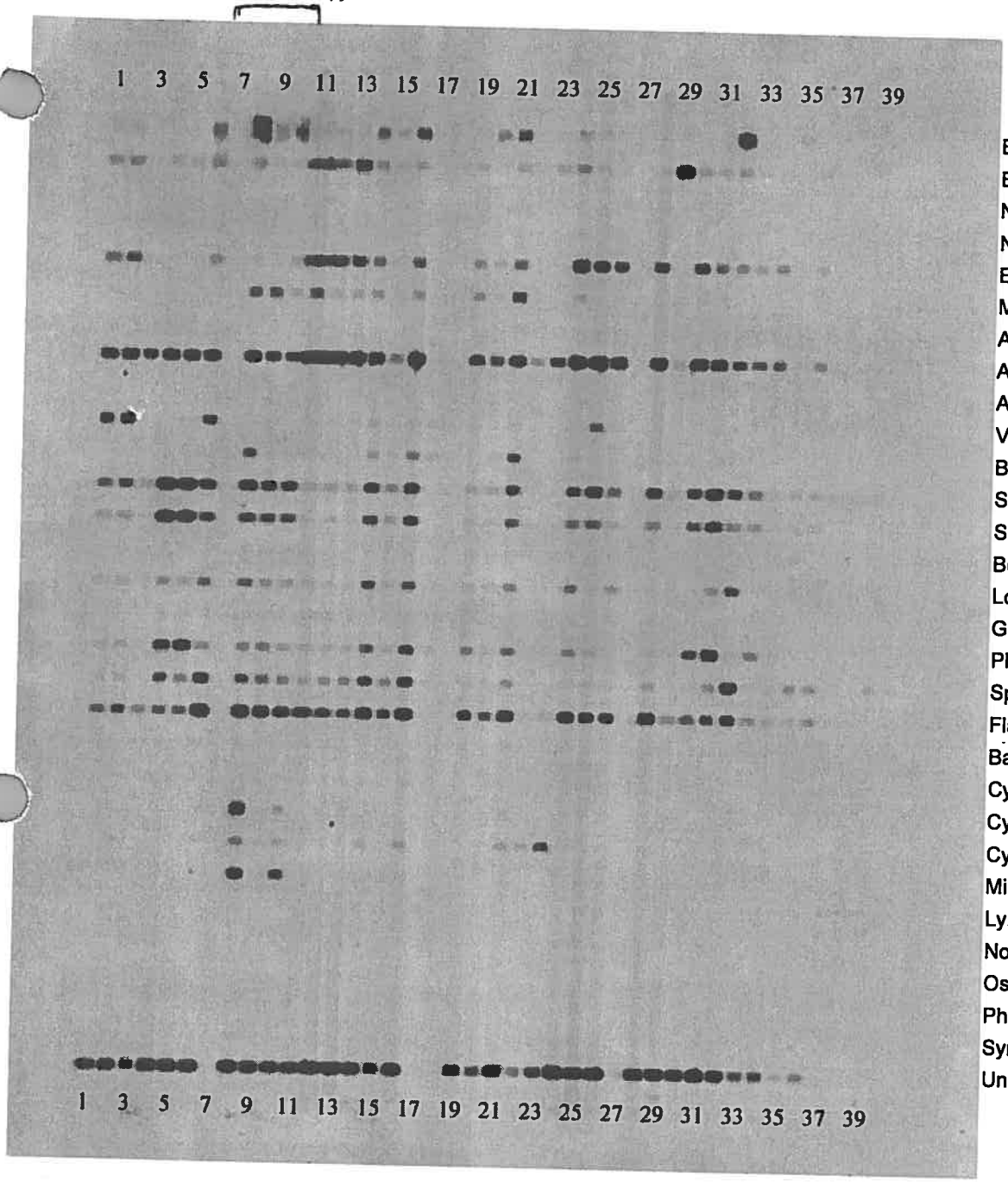



Fig 7a

Fig 7b

Checkerboard hybridization MAT LAYERS - Group I Appendix I 7/24/98




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
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]] Green
8. Group I-P2 Pink
9. Group I-G3 Gray
10. Group I-B4 Black
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