Ecological Significance, Molecular and Physiological Characterization, and Nature of a Bacterium Associated with the Heterocysts of an *Anabaena* sp. isolated from School Street Marsh, Woods Hole MA.

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ABSTRACT

Our project focused on a two-membered culture containing a cyanobacterium and a smaller organism that attached specifically to the heterocysts of the cyanobacterium. This culture was isolated from School Street Marsh during the 1997 Microbial Diversity course. Nell Ament and John Waterbury isolated the cyanobacterium from the two-membered culture. They also found that the smaller organism did not attach to all heterocyst-forming cyanobacteria. Based on this work, we set three goals. First, we addressed the significance of the attachment by attempting to re-isolate the two-membered culture from the same site a year later. Second, we began characterizing the attached organism with both molecular and physiological techniques. Finally, we wanted to ask whether the specific attachment was due to a nutritional signal or simply an effect of membrane differences.

In School Street Marsh samples from two different days we saw cyanobacteria with organisms of similar morphology attached to their heterocysts. The presence of a morphologically similar association during two different years suggests that this attachment occurs in the environment. During microscopic analysis, we noted that there were more cells attached to older heterocysts and that the cyanobacterium forms heterocysts even in the presence of combined nitrogen sources. These observations were not pursued. We also placed slides in School Street Marsh for up to three weeks before microscopic examination. While the slides were colonized by a variety of cyanobacteria, no *Anabaena*-like organisms were seen.

Molecular analysis of the two-membered culture produced two sequences closely related to *Anabaena* (94%) and two sequences related to *Zoogloea ramigera* (97%). While isolating the smaller organism we obtained organisms of three different morphologies. Two of these were characterized molecularly and were related to *Arthrobacter* sp. and *Bacillus* sp. However, we suspect that these were con aminants. Reassociation experiments with all three of these isolates were unsuccessful. Further analysis of the association requires a pure culture of the attached organism. This culturing might be facilitated by the hypothesis that this organism is related to *Zoogloea* sp.

INTRODUCTION

During the 1997 Microbial Diversity course at MBL an association was observed between a cyanobacterium and a putative prokaryote (fig. 1). The associated organisms, obtained from the School Street Marsh in Woods Hole, were isolated and initially described by Nell Ament (Ament, 1997). The cyanobacterium was identified as a species of *Anabaena* based on morphological characterization by light microscopy. Transmission electron microscopy revealed a long tapered rod associated with the cyanobacterial heterocysts with what appeared to be a prosthecae characteristic of the genus *Caulobacter*. Heterocysts are morphologically and physiologically distinct cells that are responsible for nitrogen fixation. Preliminary experimentation by Ament (Ament, 1997) indicated that the heterocyst-associated bacterium from School Street Marsh could not attach to all species of heterocyst-froming cyanobacteria. The attachment between these two organisms therefore appears to be specific.

The prosthecae of *Caulobacter* sp. are often "sticky" and can adhere to a variety of surfaces (see for example Li *et al*, 1984). In fact, *Caulobacter* were observed to attach to heterocysts during the 1993 Microbial Diversity course (G.V.N. Moorillon, 1993). There are published reports of bacteria attaching specifically to heterocysts, including one that measured an increase in nitrogenase activity in the presence of attached organisms (Lupton, 1981; Paerl, 1978; and Paerl and Kellar, 1978). However, the nature of the association between the bacteria from School Street Marsh is unknown. One explanation for the observed association could be the secretion of nitrogenous compounds from heterocysts for use by the associated organism. Another possibility is that the apparent specificity of attachment is simply an effect of membrane differences between the cell types. Based on these observations four goals were established. First, the ecological significance of the attachment was examined through an attempt to observe and isolate additional cyanobacterial associations from the same site one year later. Second, we attempted to isolate and conclusively identify the cyanobacterium and the attached organism using phylogenetic techniques based on 16S rRNA gene sequences. Third, classical enrichment techniques were employed to isolate the associated organism in pure culture. Finally, we asked whether the specific attachment was due to a nutritional signal or simply an effect of membrane adhesion. During this work isolates were obtained from School Street Marsh using a variety of enrichment techniques (summarized in Appendix 4). Water chemistry data are also included from two locations in School Street Marsh during both high and low tides (Appendix 3).

MATERIALS AND METHODS

Ecological Significance of Cyanobacterial Association

In Situ Experiments at School Street Marsh: At the front of School Street Marsh near the Eel Pond connection, glass slides were vertically positioned in a slide holder which was attached to the bottom of a plastic, wicker basket. This apparatus was secured to a metal stand and secured in the sediments adjacent to the exposed grass mats where an *Anabaena* sp. had been found prior to this experiment. The slides were immersed in

water during both low and high tide. The slides were removed at one-week intervals over a period of three weeks and viewed using phase contrast and fluorescence microscopy.

In situ experiments were also conducted at the back of the marsh near the Woods Hole Oceanographic Institute parking lot. At this location, the slide holder was secured to a plastic ice cream tub which was suspended in the water column (Newman, 1997). Samples were collected and analyzed as described for the front of marsh (see above).

Re-isolation of Cyanobacterial Associations from School Street Marsh: An attempt was also made to re-isolate cyanobacterial associations from School Street Marsh. Sediment samples were collected from a grass mat at the front of the marsh near the Eel Pond connection. These samples were placed in sterile plastic Petri dishes and placed in front of a 150 watt incandescent bulb in the laboratory. One half of the Petri dish was covered with aluminum foil to encourage growth of any cyanobacteria towards the light source and away from non-photosynthetic organisms. The green filaments that grew were examined using phase contrast microscopy.

Molecular Characterization of Cyanobacteria and Associated Organism Cultures

DNA isolation. Filaments of the pure *Anabaena sp.* and the two-membered association (obtained from Ament, 1997) grown in liquid culture (SW50) were harvested by centrifugation in 1.5 mL polypropylene tubes and DNA was extracted by bead beating followed by phenol:chloroform extraction and ethanol precipitation. DNA isolation and 16S rDNA amplification were performed according to standard methods distributed during the course. Single colonies of the associated organism were directly utilized for PCR amplification of 16S rDNA without prior purification of DNA.

16S rDNA amplification. After amplification of the 16S rDNA via PCR using universal primers (8f, 1492r), the obtained PCR products were cloned into a pCR™-TOPO vector (Invitrogen) and transformed into *E. coli*. Positive clones (white in color, indicates disruption of *LacZ* gene on the TOPO vector by insertion of the PCR fragment) were picked and the insert of the pCR™-TOPO vector amplified by PCR using TOPO primer.

Restriction Fragment Length Polymorphism (RFLP). PCR products obtained after PCR using TOPO primers were digested with *Hin*PI and *Msp*I and separated by gel electrophoresis (fig. 7). PCR products showing unique RFLP patterns were sequenced.

Checkerboard Hybridization. DNA isolated from the pure *Anabaena* sp. and the two-membered culture were used as the template in PCR reactions with dioxigenin (DIG)-labeled 16S rDNA primers (1492r and DIG-universal instead of 8f primer). Checkerboard hybridization was performed as described in materials distributed during the course.

Isolation of the Associated Organisms

In order to separate the organism attached to the heterocyst of *Anabaena sp.*, the two-membered culture obtained from Ament (1997) was incubated in C-Mix and LTY (50%) liquid and on agar plates containing the same media (Appendix 1, media recipes). The following conditions were examined in an attempt to isolate the attached organism:

1) LTY (50%): i) in tubes, without shaking
ii) in tubes, closed with rubber, reduced oxygen concentration (headspace with addition of H_2/CO_2 to generate microaerophilic conditions)
2) C-Mix: i) in tubes, without shaking

Agar plates were incubated at room temperature in the dark (to prevent growth of *Anabaena* sp.) or at 25°C with a diel cycle (14 h light/8 h dark). Liquid enrichments were incubated at 25°C with the same diel cycle. After 2 days, colonies were visible (~1-2 per plate) with a clear color and circular morphology. Light microscopy revealed a small number of organisms that remained attached to single heterocysts (no vegetative cells of *Anabaena sp.* were visible). These colonies were picked and streaked on plates containing the same media and allowed to incubate under identical conditions. After 2-3 days of incubation, colonies could not be detected in secondary transfers on agar plates. However, small colonies were observed after one week of incubation. The sal concentration of LTY (50%) was decreased (to 10%) in an attempt to stimulate the poor growth of the organism(s) associated with the heterocyst. In addition, two different media developed for the growth of *Caulobacter* sp. (PYCM and CPFF) were tested for their ability to support growth of the associated organism (Appendix 1).

Re-Association of Isolated Bacteria and Cyanobacterium

Three pure cultures obtained from the two-membered culture were tested to determine whether they would reassociate with the pure *Anabaena* sp. Cells from each colony were streaked on cyanobacteria plates (SW50 and SW50+N; Appendix 1). Filaments from the pure *Anabaena sp.* were placed on the spot of bacterial inoculation on the plate. Reassociation mixtures were incubated at 25°C on a diel cycle for five days. Each of the three isolates was tested for reassociation in liquid media (SW50).

Association of Cyanobacteria Heterocysts with Caulobacter sp.

Before a culture of the attached organism was available, we used pure *Caulobacter* sp. strains to look at attachment to the pure cyanobacterial culture. These studies were done by mixing clumps of cyanobacteria with fresh (<1 day old) cultures of *Caulobacter*. At various time points aliquots were examined for attachment. No attachment was seen to vegetative cells or heterocysts. These studies were performed by varying the age of the *Caulobacter* culture, the presence or absence of combined nitrogen, and the level of calcium. Mixtures were examined for up to three days. The strains of *Caulobacter* used in these experiments were kindly provided by Dr. Jeanne Poindexter (Barnard College).

Microscopic Examination of the Cyanobacterial Association

The two-membered culture of cyanobacterium and associated organism was examined by phase contrast, differential interference contrast (DIC), and fluorescence microscopy. Several fluorescent nucleic acids stains were used to examine the nature of the association (including acridine orange, DAPI, and rhodamine). The three non-photosynthetic bacterial isolates obtained in pure culture were also examined using similar techniques. Scanning confocal microscopy was performed on acridine orange stained cultures of the cyanobacterium and the associated organism(s).

RESULTS & DISCUSSION

Ecological Significance of Cyanobacterial Association

In Situ Experiments at School Street Marsh: Unique biofilm development was observed on slides taken from both the front and back of the marsh. Biofilms from the front of the marsh formed three distinct vertical layers on the slide; a black layer at the bottom, a purple middle layer, and a large green layer at the top. Slides from the back of the marsh were orange in color and had very little biofilm development.

Microscopic examination indicated that *Anabaena* was not present in the biofilms obtained on slides placed in School Street Marsh. Other cyanobacteria such as *Oscillatoria* sp., *Spirulina* sp., and *Phormidium* sp. were readily present in biofilms that formed on slides placed at the front of the marsh. However, at the back of the marsh few cyanobacteria were observed on slide biofilms. The absence of an *Anabaena* sp. on the slides suggests that in School Street Marsh this cyanobacteria may occupy a different habitat.

While *Anabaena* sp. were not observed in biofilm samples from the top of the water column, a putative *Anabaena* sp. was found with heterocyst-attached organisms in sediments samples removed from the grass mats at the bottom of the marsh. Microscopic examination performed on cyanobacteria obtained from sediment samples revealed a similar morphology to the two-membered culture from the 1997 course (fig.2,3). The occurrence of the *Anabaena* sp. along with the associated bacteria indicates that this association occurs in the natural environment. Attempts to isolate *Anabaena* sp. heterocyst associations were made, however growth of individual cyanobacterial filaments was too slow to obtain pure cultures during the allotted time of the course.

Molecular Characterization of Cyanobacteria and Associated Organism Cultures

Pure Anabaena sp. and Two-Membered Culture: 16S rRNA Analysis: Two different RFLP patterns were found in clones obtained by PCR amplification of the 16S rDNA from the pure *Anabaena* sp. culture (fig. 7). A representative clone with each pattern was sequenced (Appendix 2) and both demonstrated high sequence identities to *Anabaena* sp. (~94%) found in Genbank (X59559). RFLP analysis of 16S rDNA clones from the two-membered culture revealed two additional patterns (fig. 7). The sequences of these two additional clones demonstrated high similarity to *Zoogloea* sp. (X74915); the most closely related species found in Genbank was *Zoogloea ramigera* (D14255) with ~97% identity (fig. 8).

Zoogloea ramigera is a phylogenetic name applied to several Gram negative bacteria typically found in activated sludge of wastewater treatment plants and contaminated natural bodies of water. By recent convention, the species name *Z.ramigera* has been reserved for the species in the alpha-proteobacteria division of the eubacteria. This species is found as straight to slightly curved, plump, motile rods with round ends. Intracellular granules of polyhydroxybutyrate are observed under light microscopy as large refractile bodies. Cultures of *Z.ramigera* forms a viscous biofilm in liquid media at late growth stages and the cells become embedded in gelatinous matrices to form "zoogloeae." Young colonies on solid media under a normal air atmosphere are

translucent and punctiform but may increase in diameter and exhibit opaque centers. Old (>3 weeks) two-membered liquid cultures had a film over the surface of the culture and microscopically visible clumps of material containing many organisms, which is consistent with the identification of this organism within the *Zoogloea* genus.

Isolation of Associated Organisms from the Two-Membered Culture

***LTY Medium Isolates*:** Incubation of the two-membered culture on LTY medium revealed a rod shaped organism similar in shape to that observed attached to the heterocyst (fig. 4). We were unable to maintain this organism in liquid culture or on solid media, so additional characterization was not pursued. The failure to culture this organism in the absence of heterocysts could indicate that this is the heterocyst-associated organism.

***CPFF Medium Isolates*:** No growth was obtained on CPFF liquid media after 12 hr., but growth was observed after 3 days of incubation in liquid culture. Colonial morphology of isolates and microscopic examination reveal cell types similar to those obtained on PYCM medium.

***PYCM Medium Isolates*:** On PYCM liquid medium growth of a putative heterocyst-associated heterotroph was obtained in overnight cultures (with and without shaking, forming film on the surface without shaking). Microscopic examination of PYCM cultures revealed 2 -3 different cell shapes, indicating that the two-member culture contained greater than one heterocyst-associated bacterium. Alternatively, the presence of more than one bacterial morphotype could indicate contamination. The mixed culture obtained on liquid PYCM was streaked on agar plates for isolation. Single colonies were picked and individually transferred to fresh plates to obtain pure cultures. Following 4 - 5 successful transfers on PYCM agar plates, 3 isolates were obtained. Two of these isolates were molecularly characterized. RFLP patterns are shown in Figure 7 and sequences are in Appendix 2. Phylogenetic trees are shown in Figure 8.

Isolate A

> large white colonies (fig. 5)
> growth overnight at room temperature, more than 2 mm in diameter
> small rods
> change in morphology (from rods to cocci)
> 16S rDNA sequence has high identity (97%) to *Arthrobacter sp.* (D84585)

Isolate B

> tiny white colonies (fig. 5)
> growth after 2-3 days visible on plates, greater than 1 mm in diameter
> in liquid media, growth visible after 2 days (slightly turbid)
> long rods, branched filaments

Isolate C

> small yellow colonies (fig. 5)
> growth overnight, < 1mm in diameter, turns yellow after 2 days
> growth in liquid at room temperature overnight
> coccoid shape
> 16S rDNA sequence has 97% identity to *Actinomycetaceae* (isolate SR272; X87310).

Actinomycetaceae are phylogenetically and phenotypically an extremely diverse group of eubacteria. The majority of the Actinomycetaceae share a few common features: they are Gram positive and are generally non-motile in the vegetative state. This group can be coccoid, rod-shaped, or filamentous cells often branching. The presence of an *Actinomyces* sp. attached to the heterocysts is unlikely because the attached organism was Gram negative. It is likely that they are a contaminant. Since cells of similar morphology and characteristics were independently isolated three times from the original culture, these organisms were probably contaminants in the original two-membered culture.

The *Arthrobacter* group is found in the High G+C division of the eubacteria and primarily consists of Gram positive, aerobic, non-motile soil bacteria. Interestingly, species in the *Arthrobacter* group often demonstrate a coccoid-rod transition in cellular morphology during their cell cycle. An *Arthrobacter sp.* attached to the heterocysts seems unlikely because of the Gram staining and the lack of motility. This organism was therefore probably also a contaminant, but it was most likely a contaminant of the original two-membered culture as it was also independently isolated three times.

Checkerboard Hybridization

The checkerboard hybridization was performed to identify the general phylogenetic grouping of the attached organism. However, no conclusions could be drawn because the intensity of the chemiluminescence signal obtained from the two-membered sample was too low to draw any conclusions (fig. 6). Furthermore, signals from the pure *Anabaena* sp. and the association showed the same chemiluminescence signal indicating unspecific binding of the alpha-proteobacteria and flavobacteria group-specific probes.

Re-Association of Isolated Bacteria and Cyanobacterium

The three isolates obtained from the two-membered culture were examined for their ability to attach to the heterocyst in re-association experiments. Attachment of isolates to the *Anabaena* sp. heterocysts was periodically monitored by microscopy for 5 days. No evidence of re-association was found for either isolate A or isolate C. Isolate B was not tested.

If the attachment was due to a nutritional signal, it was reasoned that adding that nutrient might prevent attachment and therefore dilute out the attached cells. Growth of the two-membered culture was examined on SW50 with and without combined nitrogen source and additional nutrients (SW50+N, SW50+CY, SW50+N+CY in Appendix 1). After 1 week of incubation in the cyanoincubator, no colony formation was visible. Microscopy of the *Anabaena sp.* filaments revealed that the heterotroph is in all three cases still attached to the heterocyst and that this *Anabaena* sp. still formed heterocysts despite the fact that nitrogen sources were provided in the medium.

Additionally, no association was observed between *Caulobacter* sp. and heterocysts of the pure *Anabaena* sp. This lack of attachment was unexpected because G. V. N. Moorillon reported attachment of *Caulobacter* to *Anabaena* heterocysts (Moorillon, 1993). However, the methods used in her attachment studies were not reported in detail

and may have been very different from what was tried here. Moorillon also used a course isolate of *Caulobacter* while our *Caulobacter* strains were kindly provided by Dr. Jeanne Poindexter.

Microscopic Examination of the Cyanobacterial Association

For microscopy of the two-membered culture, slides should be made with cyanobacterial media or 80% seawater rather than with distilled water. Sealing the slides with nail polish caused more rapid lysis of the vegetative cells than sealing with paraffin (1:1 solid paraffin and paraffin oil). However, lysis still occurred and was roughly correlated to time under the microscope light rather than time on the slide. When making wet mounts, we generally crushed clumps of cells by pressing down on the cover slip or sliding the cover slip from side to side. Another method of separating the clumps was to pull them through a needle 15-25 times before making the slide. To visualize the attached organisms more clearly a variety of stains were used. DAPI worked well at 0.4 ug/mL (fig. 1) and acridine orange at 0.2 ug/mL. Dithiothreitol was used to slow bleaching but seemed to interfere with the fluorescence. We did scanning confocal microscopy on acridine orange stains of the 2-membered culture but did not see any patterns of attachment. In the future, electron microscopy might distinguish where the site of attachment is with respect to the outer layers of the heterocyst.

FUTURE WORK

Culturing the attached organism should be facilitated by the sequence information. For example, if it is a *Zoogloea* sp., published isolation procedures exist. The main difficulty is separating the cells from the matrix they produce, since other cells are often also present in that matrix. Once a pure culture of the attached organism is available and verified by reattachment, it should be possible to address the reason for the attachment. Reasons for the bacteria to attach might be a nitrogen (glutamine, arginine) or hydrogen (nitrogenase produces hydrogen during nitrogen fixation) source. The cyanobacteria might benefit by having a microenvironment that is lower in oxygen around the heterocyst. However, it is also possible that the specificity of attachment might simply be a function of the differences in membranes between heterocysts and vegetative cells, with no advantage to either organism. The first model could be tested by reassociation tests under different growth conditions while the second model predicts attachment to isolated heterocysts.

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FIGURE LEGENDS

Figure 1. DAPI stain of the 1997 two-membered culture; fluorescence microscopy with UV filter set on Zeiss Axioscope 10.

Figure 2. a,d: Phase contrast microscopy of two-membered 1997 culture. b,c: Differential interference contrast of 1998 enrichment from School Street Marsh.

Figure 3. 1998 sample obtained from School Street Marsh. a, phase contrast; b, fluorescence microscopy with UV filter set with excitation wavelength for chlorophyll A.

Figure 4. Phase contrast microscopy of LTY isolate obtained from the 1997 two-membered culture (400x magnification).

Figure 5. Phase contrast microscopy of PYCM isolates obtained from the 1997 two-membered culture (400x magnification). a & d, isolate A (*Arthrobacter*-like organism). b, isolate B (unknown). c, isolated C (*Actinomyces*-like organism)

Figure 6. Checkerboard Hybridization. Lane 22 is DNA obtained from the 1997 two-membered culture and amplified using DIG-labeled primers. Lane 23 is DNA of the pure *Anabaena* sp. isolate.

Figure 7. RFLP analysis. a, RFLP analysis of two-membered culture. b, RFLP analysis of pure isolates, lane 1-2 isolate A, lane 3 isolate C, lane 4 control *Z.ramigera*-like organism.

Figure 8. Phylogenetic analysis inferred from 16S rRNA sequence data from clones obtained from both pure isolates and two-membered culture.

Appendix 1: Media Formulations

Cyanobacteria Media

Basal media, pH 7.0

Ingredients	+N-source	-N-source
$MgSO_4x7H_2O$ (15 g/l)	5 ml	5 ml
$CaCl_2x2H_2O$ (7.2 g/l)	5 ml	5 ml
Na_2CO_3 (4 g/l)	0.5 ml	5 ml
$NaNO_3$ (30% w/v)	0.5 ml	-
NH_4Cl (1 M)	0.2 ml	-
KH_2PO_4 (6.1 g/l)	0.5 ml	0.5 ml
Na_2-EDTA (1 g/l)	1 ml	1 ml
Cyano trace metals	0.2 ml	0.2 ml
Seawater	25% (**SW25+N**)	25% (**SW25**) or 50% (**SW50**) or 50% (**SW50+N**)
Agar	+/- Agar (1%)	+/- Agar (1%)
H_2O	add 1000 ml	add 1000 ml

Media for Isolation of Heterocyst-Associated Bacteria

LTY, 50%

Basal cyanobacteria media plus addition of

Casamino acids,	0.01%
Yeast,	0.01%
Vitamin solutions (Widdel),	0.5 ml/l
50% seawater	

LTY, 10%

Basal cyanobacteria media plus addition of

Casamino acids,	0.05%
Yeast,	0.05%
Vitamin solution (Widdel),	0.5 ml/l
10% seawater	
+/- Agar (1%)	

C-Mix

Basal cyanobacteria media plus addition of

Galactose,	2.0 ml/l (5 mg/25 ml)
d-Xylose,	2.0 ml/l (5 mg/25 ml)
Arabinose,	2.0 ml/l (5 mg/25 ml)
Urea,	0.5 ml/l (5 mg/25 ml)
Vitamin solution (Widdel),	0.5 ml/l
50% seawater	
+/- Agar (2%)	

PYCM

Peptone,	0.25%
Yeast,	0.05%
$CaCl_2$,	1 mM
$MgSO_4$,	1 mM
+/- Agar (2%)	

CPFF

Casamino acids,	0.05%
Peptone,	0.05%
80% seawater	

Media for Attachment Studies

SW50+N

SW50 plus	0.01% yeast 0.01% casamino acids	= **SW50+CY**
SW50+N plus	0.01% yeast 0.01% casamino acids	= **SW50+N+CY**

Appendix 2: 16S rRNA Sequence Data

Sequences obtained from the pure *Anabaena* culture (*Anabaena sp.*)

Sequence I

TGAATTCTGGCTCAGGATGAACGCTGGCGGTACGCTTAACACATGCAAGTCGAACGGTCTTTT
CGGAGATAGTGGCGGACGGGTGAGTAACGCGTGAGAATTTGGCTTTAGGTCGGGGACAACAG
TTGGAAACGACTGCTAATACCGGATATGCCGGAAGGTGAAAGATTTATTGCCTGAAGATAAGC
TCGCGTCTGATTAGCTAGTTGGTAGTGTAAGGGACTACCAAGGCGACGATCAGTAGCTGGTCT
GAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTC
TTGGGTCGTAAACCTCTTTTCTCAAGGAAGAAAAAAATGACGGTACTkAGGAATAAGCAyGGC
TAyC

Sequence II

TCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCCTTGAGTTTGATTATGGCTCAGGATG
AACACTGGCGGTATGCTTAACACATGCAAGTCGAACGGTCTCTTCGGAGATAGTGCGGACGGG
TGAGTAACGCGTGAGAATTTGGCTTTAGGTCGGGGACAACAGTTGGAAACGACTGCTAATACC
GGATATGCCGGAAGGTGAAAGATTTATTGCCTGAAGATAAGCTCGCGTCTGATTAGCTAGTTG
GTAGTGTAAGGGACTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGG
CGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCTCTTGGGTCGTAAACCTCTTTTC
TCAGGGAAGAAAAAAAkACGGTACCkAGGAATAAGCwCGGCTAyCACT

Sequences from cloned 16S rDNA from the two-membered culture.

Sequence III

CGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTGAGTTTGATTCTGGCTCAGAACGA
ACGCTGCGGCAGGCTTAACACATGCAAGTCGAACGCATCGCAAGATGAGTGGCAGACGGGTG
AGTAACGCGTGGGAATCTACCCAACTCTACGGAACAACTCAGGGAAACTTGTGCTAATACCGT
ATACGCCCTACGGGGGAAAGATTTATCGGAGTTGGATGAGCCCGCGTTGGATTAGCTAGTTGG
TGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATT
GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC
GCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCAC
CGGAGAAGATAATGACGGTATCCGGAGAAAAGCCCGG

Sequence IV

TGAGTTkGATCATGGCTCAGAACGAACGCTGCCGGCAGGCTTAACACATGCAAGTCGAACGCA
TCGCAAGATGAGTGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCAACTCTACGGAACAA
CTCAGGGAAACTTGTGCTAATACCGTATACGCCCTACGGGGGTAAGATTTATCGGAGTTGGAT
GAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTG
GTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAG
GCCCTAGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAAAGCCCG
G

Sequences obtained from pure cultures isolated from the two-membered culture

Sequence A (*Arthrobacter sp.*)

TGGAGAGTTTGATCmTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC
GATAATGCCAGCTTGCTGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCC
TTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACTCCTCATCGCATGGT
GGGGGGTGGAAAGCTTTATTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGG
TAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAG
CCTGATGCAGCGACGCCGCGTGAGGGATGACGGCTTCGGGTTGTAAACCTCTTTCAGTAGGGA
AGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCG

Sequence C (*Actinomycetaceae*, isolate SR 272)

TGCATCGCATGGTrTGGGTTGGAAAGTTTTTCGGTGGrGGATGGGCTCGmGGCCTATCAGCTTG
TTGGTGrGGTGATGGCyTACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTT
CAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCGGG

Appendix 3. Water chemistry data for School Street Marsh; June 26, 1998.

SAMPLES SITE	DISSOLVED OXYGEN	SALINITY	NITRATE	AMMONIA	SPECIFIC CONDUCTIVITY
	mg/l	ppt	μ M	μM	mS
FRONT LOW TIDE	0.05	15.4	0.694	8.83	25.38
BACK LOW TIDE	5.85	0.1	0.712	9.14	250.0
FRONT HIGH TIDE	6.78	18.5	1.438	8.04	28.52
FRONT LOW TIDE	6.15	0.1	0.555	8.81	263.5

Water Chemistry of School Street Marsh

Water samples were collected on June 26, 1998 and analyzed for dissolved oxygen, salinity, nitrate, ammonia, and specific conductivity. A surface grab sample was collected at low and high tide from the front and back of the marsh. Samples were kept on ice in the dark. Dissolved oxygen, conductivity, and salinity were measured at the laboratory using a YSI probe. The remaining sample was filtered for the nutrient analysis.

In summary, the water chemistry data indicates a moderately saline system with low dissolved oxygen. Ammonia appeared high for early summer as it is believed to be preferentially taken up by phytoplankton compared to nitrate. The high nitrate levels in the front of the marsh at high tide suggest an influx of nitrate occurs during this time. Higher salinity values at the front of the marsh are likely due to its close proximity to Eel Pond.

Appendix 4: School Street Marsh Enrichments

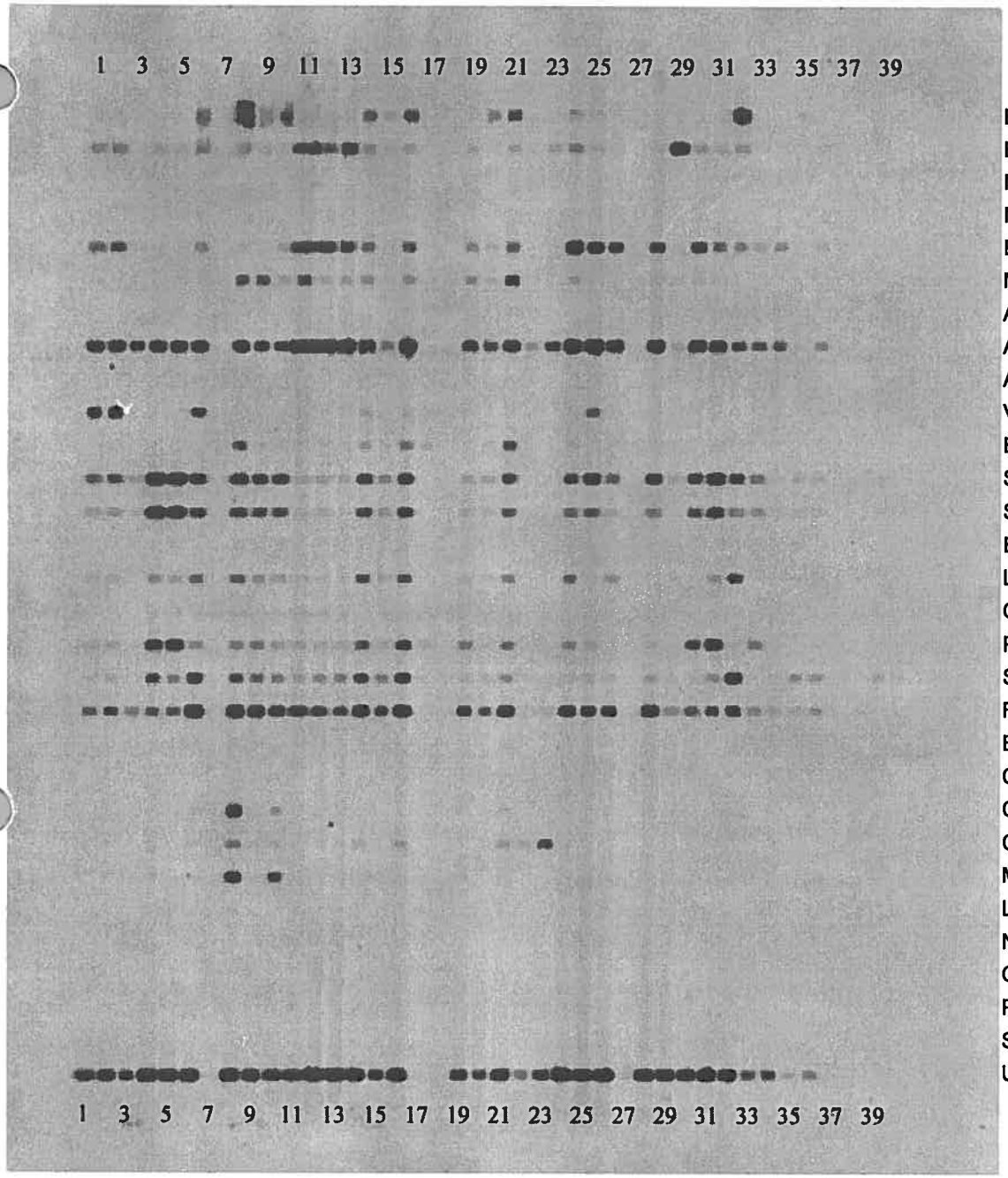
Type of Bacteria	Growth	Type of Cells	Comments	Isolates
Chemo-organotrophs:				
Benzoate + O_2 or Nitrate	+	all rods or chains of rods	23°C better than 42° C	In progress
	+	all rods	23° C better than 42° C	
Tartrate + High Nitrogen	+	cocci @ 23° C long chains of rods @ 42° C		
Tartrate + Low Nitrogen	+/-	cocci, chains @ 23° C		
Tartrate + O_2 + Low Nitrogen	-/+	long, thin rods @ 42 °C	poor growth	
Tartrate + Salt Water + Low Nitrogen			on shakes, medium-sized rods, high motility	
Isolate #6 BSWLN	-	22 °C		
	-	42 °C		
	-	42 °C		
YEN	-	42 °C		
YE YEN YE	+			
King A	+		grey/yellow colonies, no UV fluorescence	
King B			bright yellow colonies, bright UV fuorescence	
Chemolithotrophs	-			
Sporeformers:				
+/- O_2	-			
Freshwater Plates	-			
Seawater Plates	+			

Fermenting:			
Ethanol	+		acetate & propionate via HPLC
Vanillate:	+	rods, pairs of rods	
H_2O_2 + BES			
Fermenting:			
ETOH + SO_4	+	rods, curved rods	no acetate production
H_2O_2 + SO_4	+		via HPLC
Homoacetogens:			
TMA + BES	+?		acetate production via
TMA	+?		HPLC
Methanogens:	-		
Methanol + BES	+		acetate production via HPLC
Cyanobacteria	Single Cells		
Luminescent	None + (Eel Pond)		
Fe (III) Reducers:			
FeIII	+		
Fe+AQDS+Acetate	+		
Fe+Acetate	+		
AQDS+Acetate	+		
Sulfate Reducers:			
Ethanol + SO_4	+	rods, curved rods	
H_2/CO_2 /SO_4	+		

.Checkerboard hybridization

Fig 6

7/24/98

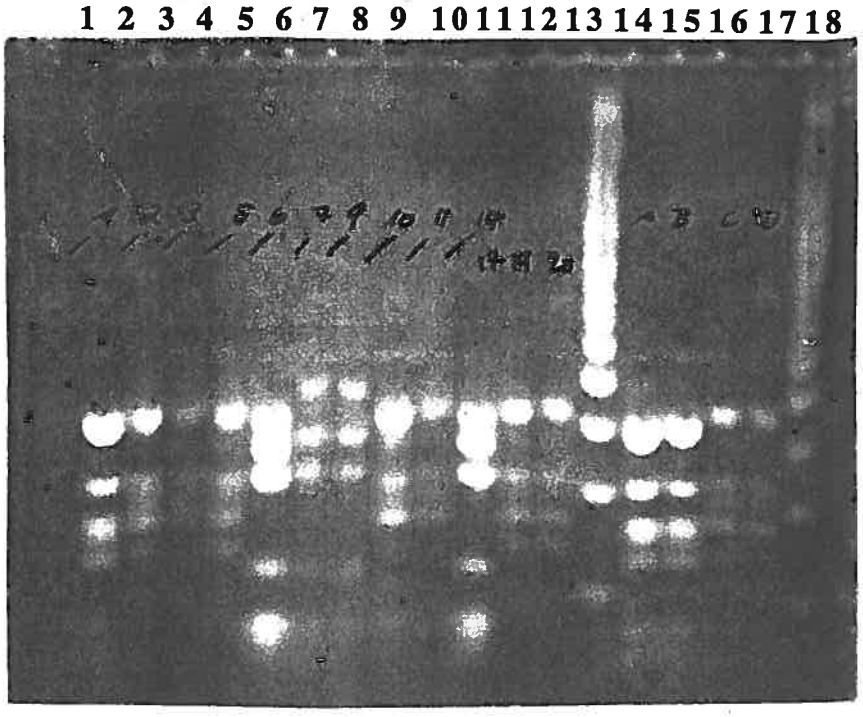


Berry "Purple Sulfur "
Betas-all
Nitrosolobus/Nitrosovibrio
Nitrosomonas spp
Enterics / some gammas
Methylotrophic bacteria
ANG Alteromonas sp.
Alphas-all
ANG Shewanella sp.
Vibrio gazogenes
Berry "Plastid"
SRB2, some deltas
SRB, some deltas
Berry SRB clones C16/C18
LoGC Gram Positive
Green Sulfur-all
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:

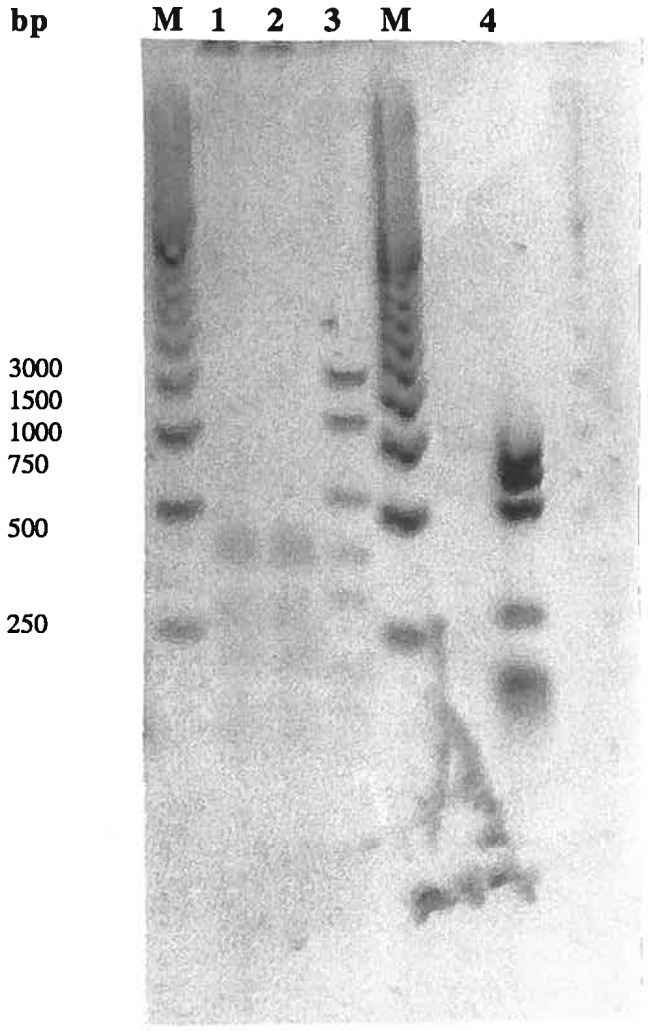
1. Silvana-salt pond 1
2. Silvana-salt pond 2
3. John oyster
4. Group I- ED
5. Group I--LD
6. Group I-MD
7. Group I-G1 [No good]]
8. Group I-P2
9. Group I-G3
10. Group I-B4
11. Milva enrich $t_0 + O_2$
12. Milva enrich $t_2 + O_2$
13. Milva enrich $t_4 + O_2$
14. Milva enrich $t_8 + O_2$
15. Milva enrichment 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

fig 7A



Lane 1-12, RFLP pattern of the two-membered culture, lane 14-17, RFLP pattern of the *Anabaena* sp.
Anabaena sp. RFLP pattern in lane: 1, 14, 15 and in lane 2, 3, 4, 8, 9, 11, 12
"Heterotroph" RFLP pattern in lane 5 and 10
in lane 6 and 7.
Lane 13 and 18, size marker

fig 7 B



Characteristics	Sporeformer Bacillus mycoides	white	yellow	pale	1	3
Cell shape	rods, in chain	long rods	short rods	rods, some in pairs	Small rods, some in pairs	rods
Spore position	terminal	terminal	terminal	terminal/ central	- spores seen	Central to terminal
PHB granules	-	+	-	-	-	+
Colony						
Color	white, milky	white, milky	pale-white	dark yellow	white	white, milky
Shape	rhizoidal	irregular, flat	circular	konvex, circular	circular	irregular, flat
Motility	-n-motile	motile	motile	-n-motile	motile	motile
Growth on						
PSW	+	+	+	+	+	+
PFW	+	+	+	+	+	+
Growth after 2nd pasteurization	+	+	+	+	+	+
Aerobic/facultative/ anaerobic	aerobic	aerobic	aerobic	aerobic	aerobic	aerobic
Growth anaerobic +/- nitrate	-	-	-	-	-	-
Starch hydrolysis##	+	+	-	-	(+)	+
growth on glucose						
Gas formation	-	-	-	-	-	-
pH without shaking#	yellow	yellow	green	green-yellow	yellow	yellow
pH with shaking#	blue	blue	green	yellow	blue	blue

as **pH indicator Bromothymolblue** was used
acidic pH: yellow
basic pH: blue
peptone media (color) is green-blue

media for **starch hydrolysis**
1% peptone, 1% starch (soluble) in tap water
detection of starch hydrolysis with iodide/potassium iodide (1%/1%) in H_2O

fig 1

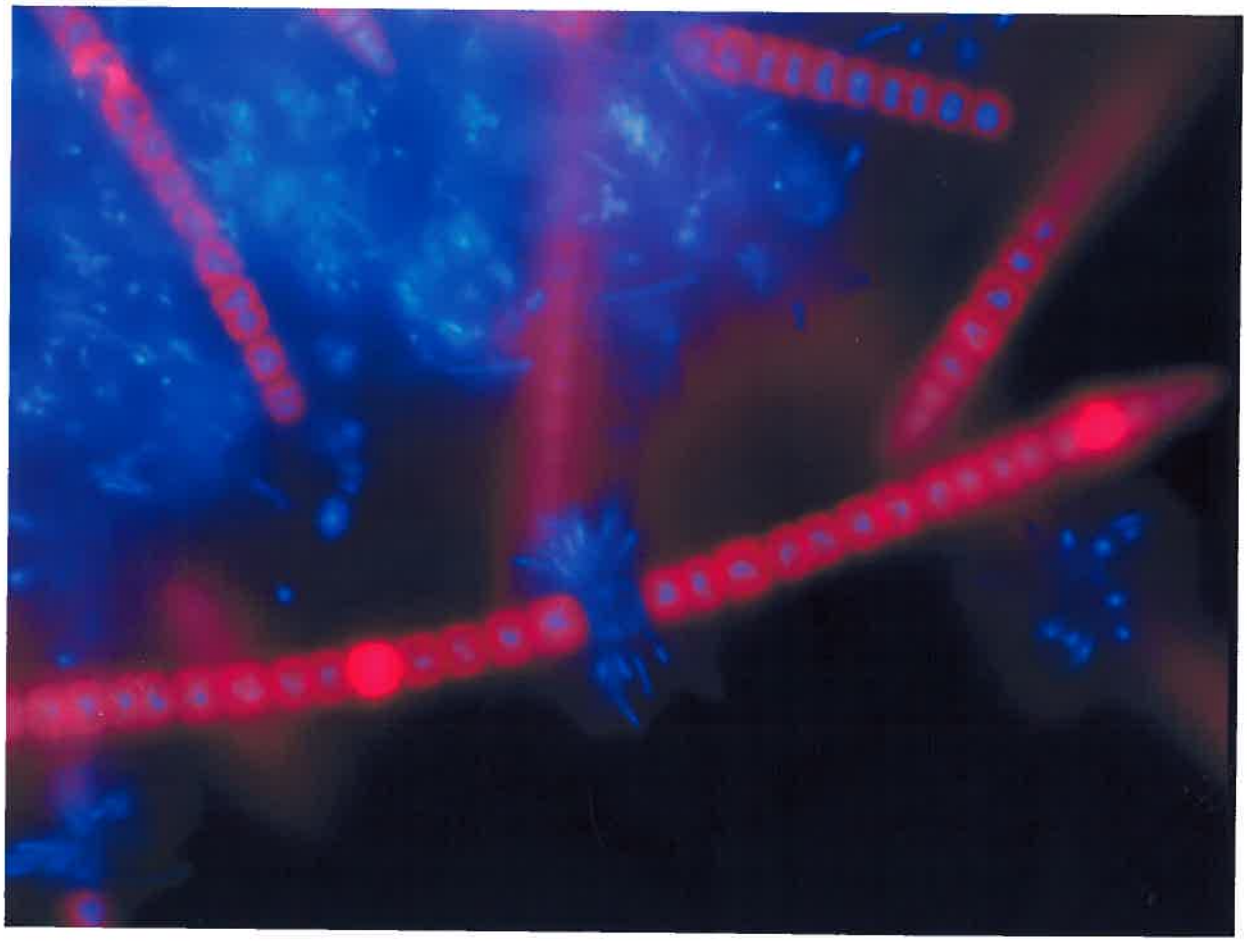


fig 2

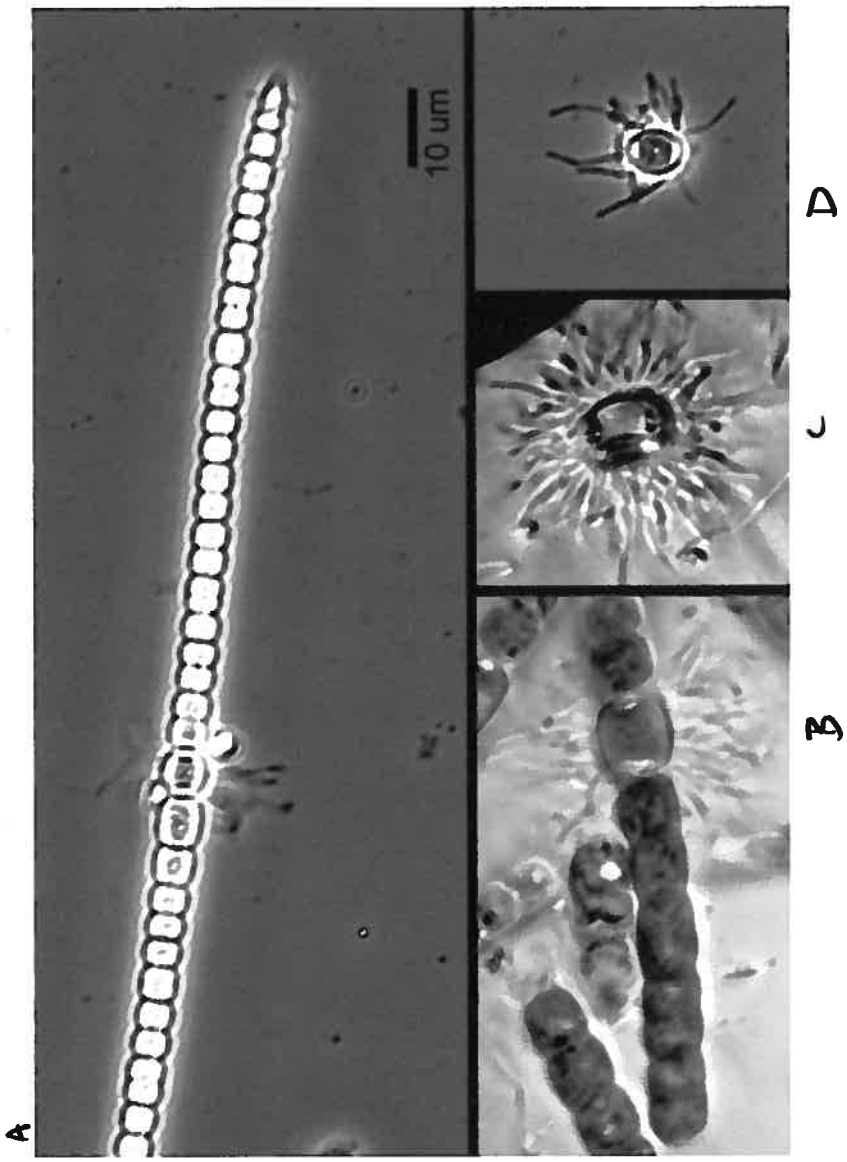
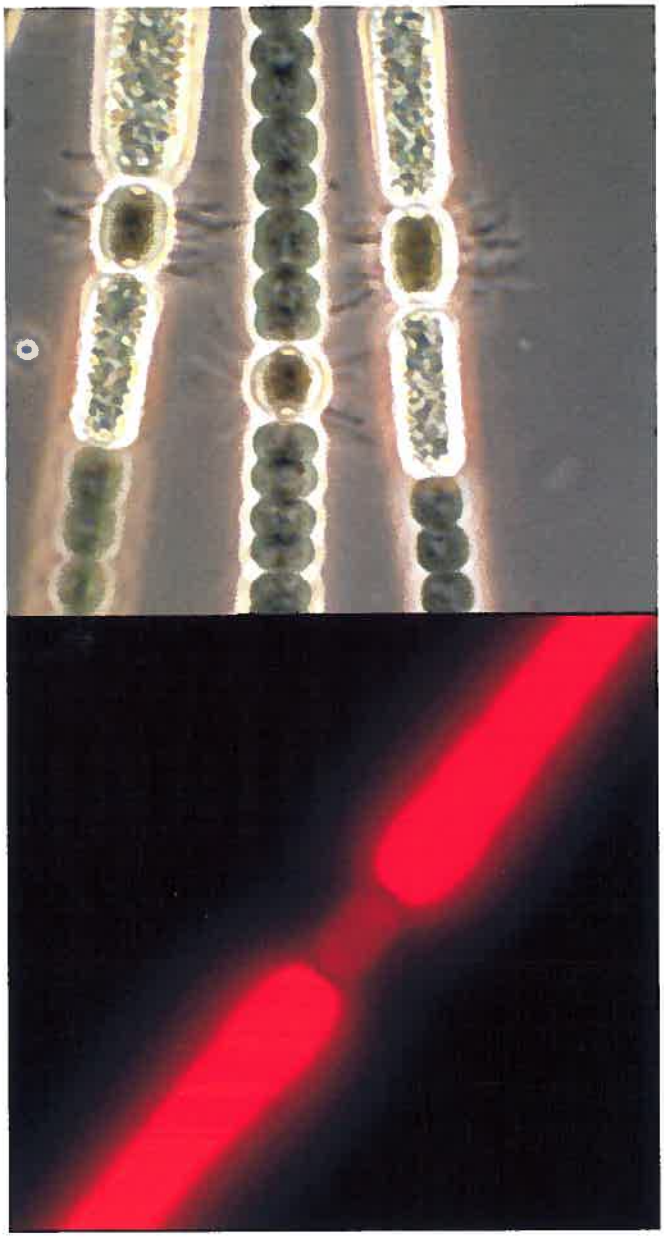


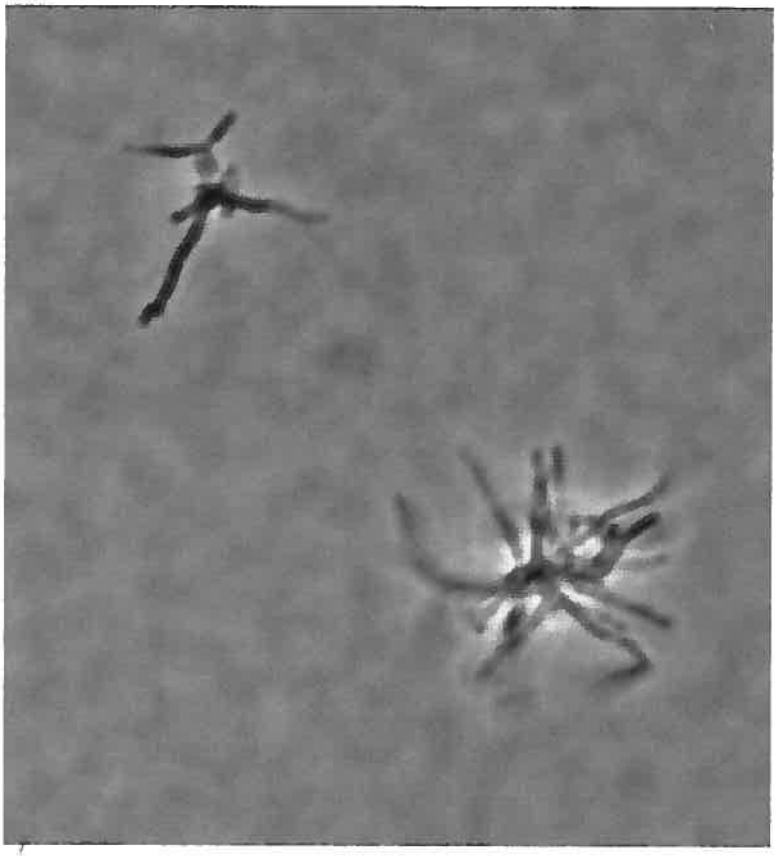
Fig 3

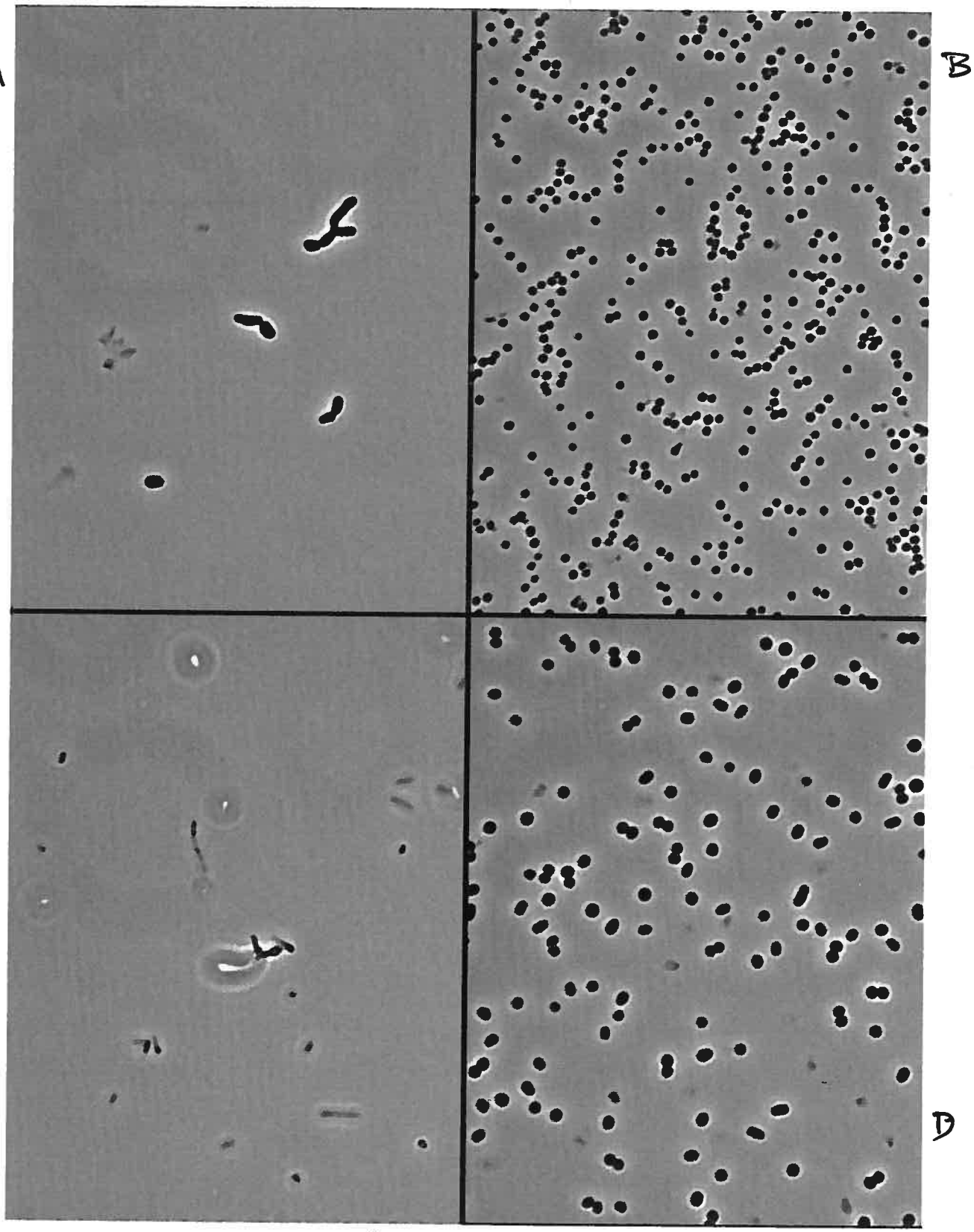


A

B

fig 4





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