Spore area

Enrichment and Identification of a Heterotrophic Epibiont Associated with an *Anabaena* sp. Isolated from School Street Marsh in Woods Hole, MA

Bradley S. Stevenson

Microbial Diversity Summer Course, 2000

A group from the Microbial Diversity course in 1997 isolated an *Anabaena* sp. from School Street Marsh in Woods Hole, MA that was seen to have bacterial epibionts strictly associated with only the cell wall of the *Anabaena* heterocysts. John Waterbury and Elinor Ament were able to isolate the *Anabaena* sp. free of its epibionts and found that the epibiont was unsuccessful at attaching to other heterocyst-forming cyanobacteria. In the 1998 Microbial Diversity course, another group amplified 16S rDNA from a culture containing the *Anabaena* sp. and the epibiont (2-membered culture). The non-*Anabaena*, amplified sequence was then determined to be most closely related to *Zoogloea ramigera*. Attempts to culture this epibiont or confirm that the "Zoogloea-like" sequence belonged to the epibiont were unsuccessful and therefore the identity of the epibiont was not confirmed. In the study reported here, this heterotrophic epibiont of the previously described *Anabaena* sp. was successfully grown aerobically in brackish, heterotrophic medium. The 16S rDNA from the *Anabaena* sp. and the epibiont was PCR-amplified and sequenced, and the resulting sequences were used to determine the preliminary phylogeny of both organisms. The heterotrophic organism grown from the 2-membered culture was confirmed to be the epibiont by microscopic analysis of the epibiont culture, its re-association with *Anabaena* sp. cells from a pure culture, and sequence comparison of 16S rDNA amplified from both the 2-member and epibiont cultures.

INTRODUCTION

A species of *Anabaena* isolated from School Street Marsh in Woods Hole, MA was found to have bacterial "epibionts" that were attached to the outer surface of the *Anabaena* sp. heterocysts (1). The identity of the epibionts remained largely undetermined until a group from the 1998 Microbial Diversity course attempted to culture the epibiont and clone its 16S rDNA gene (2). They were not successful in culturing the epibiont organism in the absence of *Anabaena* sp. cells, but a non-*Anabaena* sp. sequence was cloned from a culture containing both the *Anabaena* sp. and the epibiont (2-member culture). This sequence was identified as being closely related to that of a strain of *Zoogloea ramigera* but it was never determined whether or not this sequence represented that of the epibiont.

Associations between heterotrophic bacteria and photosynthetic cyanobacteria appear to be ubiquitous in aquatic ecosystems, and are often most pronounced during blooms. The close association between heterotrophic bacteria and the heterocysts of filamentous cyanobacteria can be species specific, and can enhance N_2 fixation (4,6,7, and 9). The heterotrophic bacteria (epibionts) enhance N_2 fixation by consuming photosynthetic O_2 in the immediate vicinity of the heterocysts (4,6,7, and 9).

In this study, the identity of both the *Anabaena* sp. and the epibiont were determined using phylogenetic analysis of 16S rDNA genes amplified and automatically sequenced using the polymerase chain reaction (PCR) with non-specific bacterial primers. The identity of the epibiont was confirmed through its growth in the absence of *Anabaena* sp. cells and its re-association with *Anabaena* sp. cells from a pure culture. Sequence analysis of 16S rDNA obtained from the *Anabaena*-free growth enrichment of

the epibiont also confirmed that the organisms in this culture were indeed the epibionts found attached to the heterocysts of the *Anabaena* sp.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

The pure culture of *Anabaena* sp. isolated from School Street Marsh and the two-member culture (*Anabaena* sp. + epibiont) were obtained from J. Waterbury. These cultures were grown in 1/2 Seawater Oligotrophic (SO) medium (Appendix A) at 25°C with a 14 h light/ 10 h dark cycle, and doubled approximately once per day. The epibiont cells were grown aerobically in 5 ml of Marine Purity (MP) medium (Appendix A) at shaking at 200 rpm and 25°C.

PCR amplification, ARDRA, and analysis of sequence phylogeny

Before PCR amplification of both the pure Anabaena sp. and the 2-member cultures, 1 ml of cells were pelleted by centrifugation, resuspended in 1% Nonidet® P40 (United States Biochemical Corp., USB), and boiled for 5 min. Cell material was then pelleted by centrifugation and the supernatant was used as the source for template DNA. Another source of epibiont template DNA was obtained by selectively collecting *Anabaena* sp. heterocysts to which the epibionts were attached (Appendix B). Vegetative cells in this culture were lysed by osmotic stress and the remaining heterocysts and attached epibionts were collected by centrifugation. DNA was extracted from this physical enrichment of epibionts using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc.). Epibiont DNA was also PCR amplified directly from the MP enrichment cultures.

PCR reactions (50 µl) were performed according to standard protocols with the eubacterial-specific forward primer SDBact0008F20 and the universal reverse primer S*Univ1391R19, an annealing temperature of 55°C, and for 30 cycles. The amplification of DNA was confirmed by electrophoresis and visualization of 5 ml of each PCR reaction on a 1.25 % agarose gel stained with GelStar nucleic acid gel stain (FMC Bioproducts).

An Amplified Ribosomal DNA Restriction Analysis (ARDRA), was used to compare the amplified products from the pure culture of *Anabaena* sp., 2-member culture, physical enrichment of *Anabaena* sp. heterocysts and epibionts, and epibiont enrichment culture. The restriction endonuclease Hpa II was used to digest 5 ml of each PCR reaction. Restriction patterns of each digestion were visualized by electrophoresis in a 2% Mataphor (FMC) agarose gel and staining the DNA fragments with GelStar (FMC) gel stain.

The sequence of amplified *Anabaena* sp. and epibiont 16S rDNA was determined using automated sequencing with fluorescent-labeled reverse primer 519R. Each sequence was first automatically aligned using the ARB software package and 16S rDNA sequence database and then further aligned manually where needed. The preliminary phylogenetic relationship of each sequence was then determined by creating a phylogenetic tree that included several related sequences using FastDNAml, a maximum likelihood algorithm.

Re-association experiment

The behavioral phenotype of the organisms growing in the epibiont enrichment culture was confirmed by a re-association experiment. In this experiment, an aliquot (50

or 100 μl) of the epibiont enrichment culture was added to a 1 ml aliquot of the pure *Anabaena* sp. culture, and incubated at 25°C with 14/10 h light cycles. Periodic microscopic analysis was used to visualize attachment of the putative epibiont to *Anabaena* sp. heterocysts.

RESULTS

Microscopic analyses

Anabaena sp. pure cultures

The *Anabaena* sp. cells were arranged in chains with 10 to 14 vegetative cells between each heterocyst and differentiated terminal cell. Occasionally dissociated heterocysts could be seen, presumably due to the lysis of dead or dying *Anabaena* sp. cells.

2-member cultures

In the 2-member cultures, there were no noticeable differences in the cellular morphology of the *Anabaena* sp. cells. Between 10 – 50 epibiont cells could be seen on each heterocyst. Attachment of the epibionts was almost exclusively on the surface of the heterocyst cell wall, focused on the junction between the heterocyst and the adjacent vegetative cells (Figure 2). Occasionally unattached epibionts could be seen, usually in some sort of extracellular matrix. No motile epibiont cells were observed in the 2-member cultures as previously reported (1).

Epibiont cultures

Aerobic growth of cells in the MP heterotrophic medium tubes was observed 7 days after inoculation with 100 µl of the 2-member culture. These cultures became very turbid after a few days more growth, with flocs forming on the bottom and at the meniscus. Cell morphology of the organisms in this culture resembled that of the epibionts seen attached to the *Anabaena* sp. heterocysts in the 2-member culture (Figure. 3). The putative epibionts were irregularly-shaped, branched rods that were enlarged at one end and appeared to have a *Caulobacter*-like holdfast at the opposite end. Motile cells were observed in the enrichment culture. Cell motility was awkward, presumably due to cell morphology. Cells in the enrichment culture were observed to attach to one-another, forming rosettes and bundles (Figure 3).

Re-association experiments

More epibiont cells were present in the re-association experiments relative to the 2-member culture (Figure 4). This could have been due to either the large inocula used to promote attachment or the continued growth of unattached epibionts in the experimental sample. The epibionts did however have a similar affinity for attaching to the heterocysts over the vegetative cells of the *Anabaena* sp. (Figure 5).

PCR amplification and phylogenetic analysis of 16S rDNA sequences

When the prepared samples of both the pure *Anabaena* sp. and the 2-member culture were used for PCR amplification, ARDRA analysis indicated no difference

between the amplified products (data not shown). This was taken as an indication that either only the *Anabaena* sp. DNA was being amplified or that both cultures also contained the epibiont. Further microscopic analysis of the pure *Anabaena* sp. culture suggested that it was still a pure culture and therefore no epibiont DNA was being amplified. In 2-member culture samples, microscopic analysis showed that neither the heterocysts nor the epibionts were affected by the method used to liberate DNA from the cells. When the heterocysts and attached epibionts were first physically enriched and DNA was extracted using the MoBio DNA extraction kit, PCR amplified product gave a different ARDRA banding pattern (Figure 6), presumably because the epibiont 16S rDNA was now being amplified.

The nucleotide sequence of PCR amplified 16S rDNAs from the enriched heterocysts and attached epibionts as well as the epibiont enrichment culture had identical ARDRA patterns (Figure 7), identical sequences (Apendix C), and both were nearly identical to two independently cloned 16S rDNA fragments amplified and cloned in 1998 (2). When these sequences were used in a BLAST search, they came up to be over 97% similar to *Zoogloea ramigera* (data not shown).

After proper alignment of these sequences against closely related sequences in the ARB database, a maximum likelihood phylogenetic tree was built with closely related sequences. According to these results, the epibiont 16S rDNA sequences belong in the α-proteobacterial group, among the rhizobia and agrobacteria (Figure 8). There are several sequences more closely related to the *Z. ramigera* sequences that are placed in the same group. These sequences correspond to an organism that has historically been considered a strain of *Z. ramigera* but has recently been proposed to

be a misnamed *Rhizobium* sp. (5 and 10). The other two recognized *Z. ramigera* strains are β-proteobacteria and likely represent two different species or even genera. As expected, the sequence representing the *Anabaena* sp. 16S rDNA was most closely related to other cyanobacteria (Figure 9).

DISCUSSION

Results of the study reported here implicate a previously unknown α-proteobacterium related to *Rhizobial* and *Agrobacterial* species as the epibiont found specifically attached to a species of *Anabaena* isolated from School Street Marsh in Woods Hole, MA. A group from the 1998 Microbial Diversity course successfully cloned the 16S rDNA sequence of this organism and determined that it was closely related to a strain of *Z. ramigera* (2). This identification was tentative at best since there was no further evidence that this sequence represented that of the epibiont. *Zoogloea* are characteristically similar to *Pseudomonas* species, which are common laboratory contaminants and therefore certainly might not be the epibiont. The similarity in sequence between the epibiont and *Z. ramigera* was also potentially misleading based on the fact that the three organisms traditionally thought to be different strains of *Z. ramigera* represent different genera and species (5 and 10).

Traditionally, the classification of organisms as *Zoogloea* was largely based on the formation of zoogloeal flocs of cells in an exracellular, gelatinous matrix. This phenotypic characterization is largely to blame for the fact that the *Z. ramigera* sequence closely related to that of the epibiont is that of an α-proteobacterium that is related to *Rhizobia* and *Agrobacteria* species, whereas the other two *Z. ramigera*

species are β-proteobacteria (5). This case is a very good example of the potentially misleading information obtained from a BLAST search, which is based on sequence similarity. A phylogenetic analysis of the sequence provided the additional information that this sequence was related to the *Rhizobia* and *Agrobacteria* found in the α-proteobacteria (Figure 8).

Although not a pure culture, the aerobic, heterotrophic growth of cells that are morphologically similar to the epibionts provided multiple pieces of evidence supporting the identification implication of this organism (Figure 3). Cells with different morphologies were not seen during microscopic analysis, only one ARDRA pattern (Figure 7) and sequence was amplified from these putative epibiont cultures, but it would be premature to consider these pure cultures of the epibiont. However, the same 16S rDNA sequence was obtained from the heterocyst-epibiont physical enrichments and the epibiont cultures (Figure 8). Additionally, cells from the epibiont cultures were seen to attach preferentially to the *Anabaena* sp. heterocysts during re-association experiments with the pure *Anabaena* culture (Figure 4 and 5).

The close phylogenetic relationship between the epibiont sequence and *Rhiziobia* and *Agrobacteria* species sequences might be used to suggest the metabolic properties of the epibiont as well as the nature of the relationship between the *Anabaena* heterocysts. These closely related α-proteobacteria are known for their close association with plants, either symbiotic, parasitic, or pathogenic. Many members of this group of bacteria can fix nitrogen, are chemotaxic towards plant root exudates, and have variable cellular morphologies. Do the epibionts identified in this study remove photosynthetic O_2 from the close vicinity of the heterocysts, enhancing

nitrogenase activity? Are the epibionts taking advantage of the reduced environment around the heterocyst to allow them to better fix nitrogen on their own? Are the epibionts fixing nitrogen, using exudates from the heterocysts, and also providing the *Anabaena* cells with a source of fixed nitrogen?

Future directions of the work presented here could include investigations into the nature of the relationship between the epibionts and hetereocysts, possibly answering the questions stated above. Much more information could be gained through comparative studies among the closely related organisms that are currently present in culture collections. A better understanding of the phylogenetic relationship among these organisms could be developed after sequencing their complete 16rDNA genes. This one relatively clear answer about the identity of this *Anabaena* sp. heterocyst epibiont has provided the basis for many more questions about its physiology and ecology.

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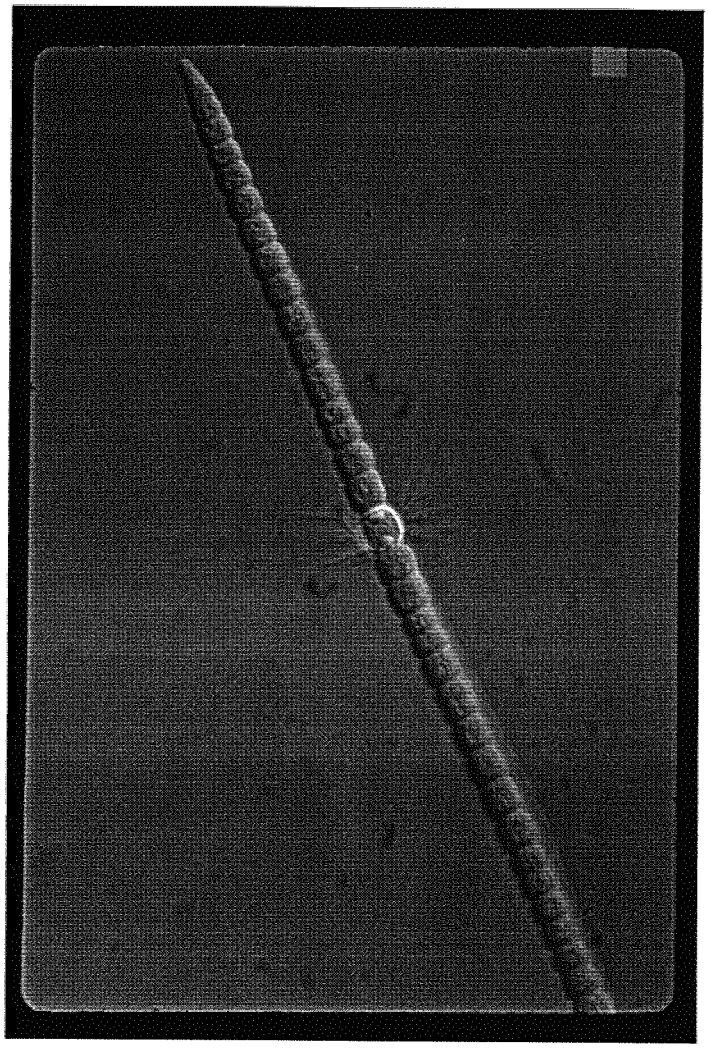

Figure 2. DIC image (1000x) of *Anabaenae* sp. with epibionts attached to the heterocyst.

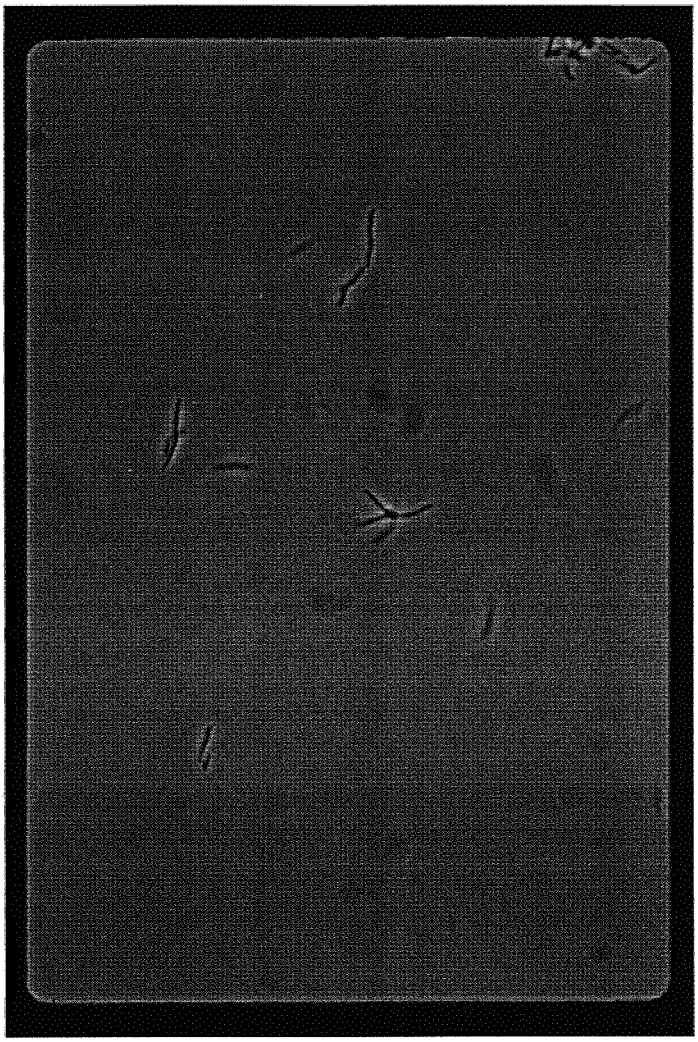

Figure 3. Phase contrast image (1000x) of the epibiont culture showing branching cellular morphology and the tendency of epibiont cells to adhere to one another.

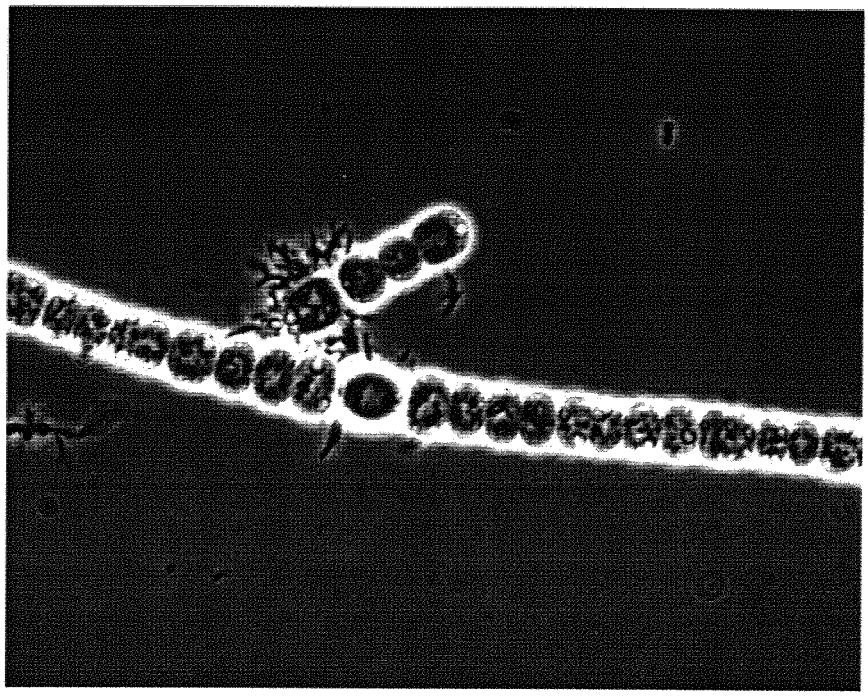

Figure 4. Phase contrast image (1000x) of re-association experiment. Cells from the epibiont culture are shown attaching to the Anabaena sp. heterocysts from a pure culture.

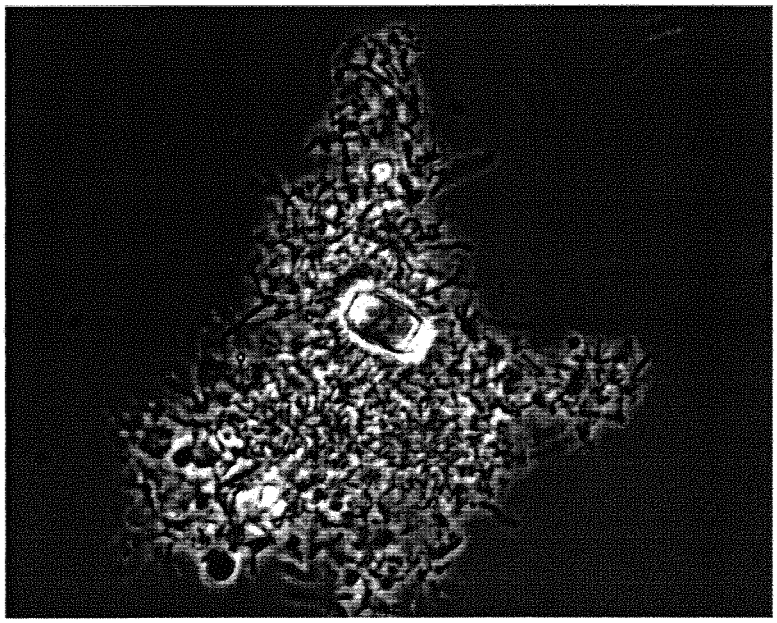

Figure 5. Phase contrast image (1000x) of epitionts attached to isolated Anabaena sp. heterocyst in re-association experiment.

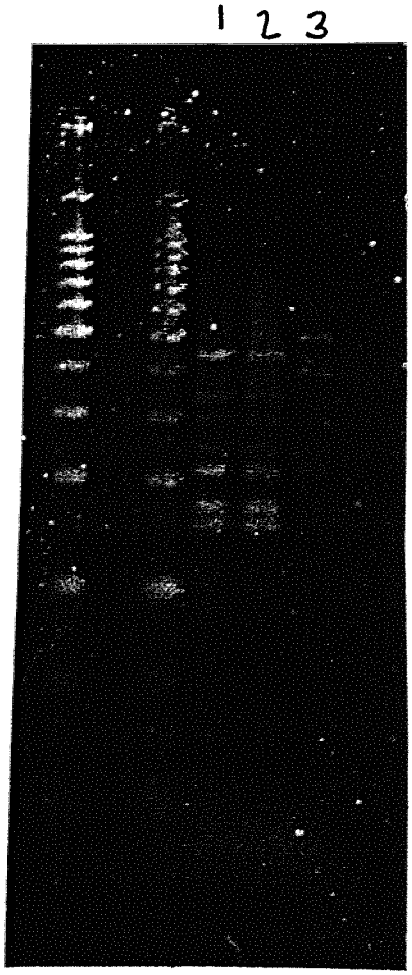

Figure 6. ARDRA patterns of Anabaena sp. amplicon (lanes 1 and 2) from Anabaena pure culture and 2-member culture, respectively. Epibiont 16S rDNA showing a different pattern is in lane 3.

Appendix A

Media Composition (J. Waterbury)

1/2 Saltwater Oligotrophic (SO) medium

Double distilled H_2O	250 ml
Filtered seawater	750 ml
Na_2CO_3	50 µM
K_2HPO_4	50 µM
Cyano trace metals	0.5 ml
EDTA (disodium salt)	7.5 µM

Cyano trace metals

$ZnSO_4 \cdot 7H_2O$	0.222 g/L
$MnCl_2 \cdot 4H_2O$	1.40 g/L
$Co(NO_3)_2 \cdot 6H_2O$	0.025 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.39 g/L
Citric Acid Hydrate	6.25 g/L
Ferric Ammonium Citrate (brown crystals)	6.0 g/L

Marine Purity (MP) medium, 1 liter

NaCl	20 g
AC broth (Difco)	17 g
$MgSO_4 \cdot 7H_20$	8.0 g
$CaCl_2 \cdot 2H_2O$	0.6 g

Dissolve $MgSO_4$ and $CaCl_2$ each seperately with dH_20. Autoclave NaCl/AC broth, $MgSO_4$, and $CaCl_2$ solutions and combine after cooling to < 65°C to avoid precipitate formation.

Appendix B

Method for physical enrichment of Anabaena sp. heterocysts and attached epibionts

11. Add 0.5 ml 50% glycerol to 0.5 ml aliquit of 2-member culture sample, incubate at room temp for 20 min.
12. Pellet cells by centrifugation in microcentrifuge at 14,000 rpm, 1 min. Decant supernatant.
13. Resuspend pelleted cells in 1 ml 10 mM EDTA

1. vegatative cells will have mostly lysed due to osmotic shock
2. observe degree of lysis under microscope

14. Pellet intact hetercysts and attached epibionts by centrifugation at 3, 000 rpm for 1 min. Decant supernatnat (will be blue-green die to phycocyanin). Resuspend pellet in 1% SDS to further lyse any remaining vegetative cells.
15. Pellet heterocysts and attached epibionts by centrifugation at 8,000 rpm for 1 min. Decant supernatant, resuspend in 0.2 ml dH_2O.

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     1          11         21         31         41         51         61         71         81         91        100
     |          |          |          |          |          |          |          |          |          |          |
   1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 0     HetEpi
   1 CGGCCGCCAG TGTGATGGAT ATCTGCAGAA TTCGCCCTTG AGTTTGATT- CTGGCTCAGA ACGAACGCT- GCGGCAGGCT TAACACATGC AAGTCGAACG  98  1998seq1
   1 .......... .......... .......... .......... A-UUUGAU-C CUGGCUCAGA ACGAACGCUG GCGGCAGGCU UAACACAUGC AAGUCGAACG  58    Epicul

     101        111        121        131        141        151        161        171        181        191        200
     |          |          |          |          |          |          |          |          |          |          |
   1 .......... .......... .......... .ACGCGUGGG AAUCUACCCA ACUAUACGGA ACAACUCAGG GAAACUUGUG UUAAUACCGU AUACGCCAUA  69     HetEpi
  99 CATCGCAAGA TGAGTGGCAG ACGGGTGAGT AACGCGTGGG AATCTACCCA ACTCTACGGA ACAACTCAGG GAAACTTGTG CTAATACCGT ATACGCCCTA 198  1998seq1
  59 CAUCGCAAGA UGAGUGGCAG ACGGGUGAGU AACGCGUGGG AAUCUACCCA ACUCUACGGA ACAACUCAGG GAAACUUGUG CUAAUACCGU AUACGCC-UA 157    Epicul

     201        211        221        231        241        251        261        271        281        291        300
     |          |          |          |          |          |          |          |          |          |          |
  70 CGGGGGAAAG AUUUAYCGGA N-UUGGAUGA GCCCGCGUUG GAUUANCUAG UUGGUGGGGU AAAGGCCUAC CAAGGCGACG AUCCAUAGCU GGUCUGAGAG 168     HetEpi
199 CGGGGGAAAG ATTTATCGGA -GTTGGATGA GCCCGCGTTG GATTAGCTAG TTGGTGGGGT AAAGGCCTAC CAAGGCGACG ATCCATAGCT GGTCTGAGAG 297  1998seq1
158 CGGGGGAAAG AUUUAUCGGA -GUUGGAUGA GCCCGCGUUG GTUUAGCUAG UUGGUGGGGU AAAGGCCUAC CAAGGCGACG AUCCAUAGCU GGUCUGAGAG 256    Epicul

     301        311        321        331        341        351        361        371        381        391        400
     |          |          |          |          |          |          |          |          |          |          |
169 GAUGAUCASC CACAUUGGGA CUGAGACACG GCCCAAACUC CUACGGGAGG CAGCAGUGGG GAAUAUUGGA CAAUGGGCGC AAGCCUGAUC CAGCCAUGCC 268     HetEpi
298 GATGATCAGC CACATTGGGA CTGAGACACG GCCCAAACTC CTACGGGAGG CAGCAGTGGG GAATATTGGA CAATGGGCGC AAGCCTGATC CAGCCATGCC 397  1998seq1
257 GAUGAUCAGC CACAUUGGGA CUGAGACACG GCCCAAACU- CUACGGGAGG CAGCAGUGGG GAAUAUUGGA CAAUGGGCGC AAGCCUGAUC CAGCCAUGCC 355    Epicul

     401        411        421        431        441        451        461        471        481        491
     |          |          |          |          |          |          |          |          |          |
269 GCGUGAGUGA UGAAGGCCCU AGGGUUGUAA AGCUCUUUNC ACCCGGAGAA GAUAAUGACG GUAUCCGGAG AAGAAGCCCC GGCUAACUUA C           359     HetEpi
398 GCGTGAGTGA TGAAGGCCCT AGGGTTGTAA AGCTCTTT-C AC-CGGAGAA GATAATGACG GTATCCGGAG AA-AAGCCC- GG........ .           475  1998seq1
356 GCGUGAGUGA UGAAGGCCCU AGGGUUGUAA AGCUCUUU-C A......... .......... .......... .......... .......... .           395    Epicul
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Appendix C