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**Genes Involved in Diazotrophic Growth of *Anabaena* sp. PCC 7120**

**A Thesis Submitted to the Graduate Division of the University of Hawai'i in Partial  
Fulfillment of the Requirements for the Degree of**

**Master of Science**

**In**

**Microbiology**

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**By**

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We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Microbiology.

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# CHAPTER 1

## Introduction

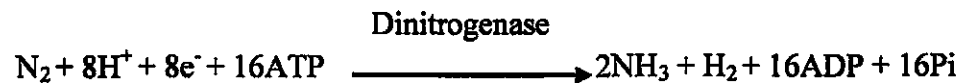
### **Cyanobacteria and *Anabaena***

Cyanobacteria, earlier known as blue green algae, are classified under the domain eubacteria. They are capable of both photosynthesis and nitrogen fixation. They have the oldest fossil record, dated 3.5 billion years old, indicating their role in the production of oxygen on earth *via* photosynthesis. *Anabaena* sp. PCC 7120 is a filamentous cyanobacterium. In the presence of fixed nitrogen, usually nitrate or ammonium, all the cells in the filament are vegetative cells. In the absence of fixed nitrogen, vegetative cells differentiate into heterocysts at semiregular intervals. Thus, a one dimensional developmental pattern of single heterocysts separated by 10-15 vegetative cells is established in fixed nitrogen free environment (18). This is one of the simplest as well as oldest examples of multicellular pattern formation in developmental biology.

### **Nitrogen fixation**

#### *Significance of nitrogen fixation:*

The term nitrogen fixation means the reduction of  $N_2$  to  $NH_4^+$ . Microorganisms play a significant role in fixing nitrogen. The total biological nitrogen fixation ( $175 \times 10^{12}$  g/year) is twice as much as total non-biological nitrogen fixation ( $80 \times 10^{12}$  g/year) (3).

***Biological nitrogen fixation:***

For nitrogen fixation to take place in heterocysts, in addition to the dinitrogenase enzyme, a source of reductant, a supply of ATP and a mechanism for the removal of  $\text{NH}_4^+$  are necessary (15). The reductant required is presumed to be produced by metabolism of sucrose *via* the oxidative pentose phosphate pathway. ATP is formed from cyclic photophosphorylation using photosystem I and from the respiratory electron transport chain. Ammonium is assimilated into glutamine and transported to vegetative cells (41)

***Nitrogenase:***

The enzyme that catalyses biological nitrogen fixation, nitrogenase, has two components - component I and component II. Component I, termed dinitrogenase, is a MoFe protein. Component II is an Fe protein, termed dinitrogenase reductase (27). Dinitrogenase is encoded by *nifD* and *nifK*, whereas, dinitrogenase reductase is encoded by *nifH*. This enzyme complex is inactivated in the presence of oxygen. Hence, in *Anabaena*, heterocysts evolved to protect the nitrogenase enzyme complex from oxygen.

## Heterocyst formation

### *Role of heterocysts:*

In *Anabaena*, under nitrate-/ammonium-depleted conditions, heterocysts are the sites of nitrogen fixation. Oxygen evolved in the vegetative cells during photosynthesis makes nitrogenase non-functional. Hence, spatially separated heterocysts are formed in order to protect nitrogenase. Heterocysts are adapted morphologically, metabolically and genetically from the vegetative cells to create an ideal condition for the activity of nitrogenase.

### *Morphological changes:*

Heterocysts are larger than vegetative cells. They have two additional layers of envelope made of polysaccharides and glycolipids. Glycolipids, the unique innermost layer of cell envelope, provides a hydrophobic barrier against the entry of oxygen. Genes *hglD*, *hglE* and *hglC* are involved in glycolipid formation (24). A polysaccharide layer protects the glycolipid layer from physical damage (56). Some of the genes that are involved in polysaccharide formation are *hepA*, *hepB*, *hepC* and *hepK*. The inner layer to the glycolipids is made of peptidoglycan, which is thicker than that in most Gram-negative bacteria and is present in both vegetative cells and heterocysts. The enzymes that synthesize and remodel the peptidoglycan are known as penicillin binding proteins (PBPs). A *pbpB* mutant of *Anabaena* obtained as the result of transposon mutagenesis was incapable of fixing atmospheric nitrogen under aerobic conditions (28). Recently it was found that *pbp6*, another *pbp* homologue in

*Anabaena* is essential for diazotrophic growth under aerobic conditions (29). Hence, even though the peptidoglycan layer is not unique to heterocysts, the presence of this layer is absolutely necessary for the aerobic nitrogen fixation.

*Metabolic changes:*

Various changes are made in the metabolic activities to reduce the oxygen content inside the heterocysts. (i) Heterocysts have an increased respiration rate to consume the oxygen molecules that still enters through the cell-envelope (56). (ii) Photosystem II which is responsible for the production of oxygen is absent in heterocysts (52), (50), (31)). (iii) Phycocyanin, a light harvesting pigment, is degraded in heterocysts. (iv) Proteases specific to heterocysts are produced. They are involved in the degradation of proteins involved in photosystem II and many other vegetative cell proteins. They remove the damaged or inactivated polypeptides. Thus, proteases contribute to continuous recycling of amino acids for synthesis of new proteins to be made in developing heterocysts (45).

*Genetic changes:*

Changes at the genetic level occur mainly during heterocyst formation and nitrogen fixation. During the process of conversion of a vegetative cell into a heterocyst, there are two main steps – decision-making and differentiation. In the decision-making step, the would-be heterocysts are determined. Genes like *ntcA*, *hetR*, *hetC*, *patS*, are involved in the decision making step. NtcA belongs to cAMP receptor protein (CRP) family and is involved in heterocyst differentiation (22). This protein senses the

nitrogen deprivation in the cells and activates the expression of *hetR*. HetR, a serine protease (57), is the master regulator involved in heterocyst formation and pattern formation (5, 7). Overexpression of *hetR* on a multicopy plasmid (7, 8) leads to formation of multiple contiguous heterocysts (Mch) even in presence of fixed source of nitrogen. However, *hetR* deletion mutant fails to produce heterocyst. HetR homodimer binds to the promoter region of *hetR*, *patS* and *hepA* genes (21). The *patS* gene, a negative regulator involved in heterocyst pattern formation, encodes a small peptide inhibitor of heterocyst differentiation (18) whereas *hetN*, another negative regulator involved in maintenance of spacing, encodes a ketoacyl reductase (4). PatS, works by lateral inhibition, produced in the early proheterocysts inhibits the differentiation of the neighboring cells, thus establishing a pattern (18). Expression of *hetN* is primarily in heterocysts. HetN is presumed to be active in mature heterocysts and inhibit neighbouring vegetative cells from differentiation (10). HetC, a protein which has similarity to ATP-binding cassette (ABC) membrane transport proteins, is involved in early stage of heterocysts differentiation. Thus, *hetC* mutant is unable to form heterocysts (26).

In the differentiation step, morphological and metabolic changes take place in the vegetative cells to form heterocysts (49). Hence, genes involved in the differentiation step are related to formation of glycolipids and polysaccharide layers. Genes involved in nitrogen fixation are generally called *nif* genes. *nif* genes occur in a cluster in the *Anabaena* chromosome. Some of the *nif* genes in the cluster are *nifB*, *nifS*, *nifU*, *nifH*, *nifD*, *nifK*, *nifE* and *nifN*. The operon *nifHDK* encodes the nitrogenase enzyme complex. It is towards the end the differentiation process that the

11kb intervening fragment is excised from the *nifD* coding region and active *nifHDK* operon is formed (17).

## CHAPTER 2

### **FraG is necessary for filament integrity and heterocyst maturation in the cyanobacterium *Anabaena* sp. PCC 7120**

(A major portion of this chapter has been submitted to the Microbiology for publication)

In *Anabaena*, in the absence of fixed form of nitrogen, vegetative cells differentiate into nitrogen-fixing heterocysts at semiregular intervals along the filament. Heterocysts can be distinguished from vegetative cells microscopically by their larger size and thicker cell envelope. In order to create microaerophilic conditions for the activity of nitrogenase, they have two additional layers of envelope made of polysaccharides and glycolipids (34). The glycolipid layer, which is the innermost of the two, provides a hydrophobic barrier against the entry of oxygen (46, 48). The exterior polysaccharide layer is thought to preserve the integrity of the glycolipid layer. Genes necessary for the production and localization of both layers have been found and pathways for their synthesis have been proposed (13, 20). Once a microaerophilic environment has been created inside the heterocysts, they fix atmospheric nitrogen and transport it to vegetative cells and in return receive a source of reductant required for fixation from vegetative cells (42).

The pattern of heterocysts along a filament is determined by the interplay of positive and negative acting regulatory factors, and approximately 12 hours after the removal of fixed nitrogen, select cells have committed to terminal differentiation into heterocysts (31, 50, 56). PatS and HetR appear to be the two central factors that control differentiation and pattern formation. HetR is the master regulator and has

both DNA-binding and protease activity (7, 21, 57). It displays positive autoregulation and expression of *hetR* is induced in proheterocysts prior to commitment to differentiation (5).

In order to identify the genes involved in diazotrophic growth and differentiation of heterocysts by *Anabaena*, a genetic screen was conducted to isolate mutants incapable of growth in the absence of fixed nitrogen. Interruption of the coding region of *fraG*, the gene upstream of *hetR*, by a transposon resulted in a fragmentation mutant that was unable to grow in the absence of a fixed source of nitrogen. The predicted protein is similar to permeases and is necessary for filament integrity and maturation of heterocysts to the point of glycolipid layer formation.

The enzymes that synthesize and remodel the peptidoglycan are generally known as penicillin binding proteins (PBPs). In *E. coli*, 12 PBPs have so far been identified, of which only PBPs 1a and 1b are essential for cell viability (54). In *Anabaena*, peptidoglycan is present inner to the glycolipid layer. A *pbpB* mutant of *Anabaena* was incapable of fixing atmospheric nitrogen under aerobic conditions (28). This mutant in the presence of fixed nitrogen did not show any significant difference in the phenotype with respect to that of the wild type. But, in the absence of a fixed form of nitrogen, filaments were yellow, short and twisted. Vegetative cells were unequal in size and shape. Heterocysts were distorted with thin envelopes and with no cyanophycin granules at the poles. In the genetic screen described, a *pbp6* mutant that was unable to fix atmospheric nitrogen under aerobic conditions was obtained. The predicted protein consists of both the transglycosidase as well as

transpeptidase domains that might be involved in the formation of peptidoglycan in *Anabaena*.

## MATERIALS AND METHODS

**Strains and culture conditions.** Table 1 describes the *Anabaena* strains, plasmids and oligonucleotides used in this study. The wild-type strain, *Anabaena* sp. PCC 7120, and its derivatives were grown in BG-11 medium, and for induction of heterocysts they were transferred to BG-11<sub>0</sub> medium as previously described (6). Transposon mutagenesis, screening for mutants unable to grow diazotrophically and recovery of the transposon insertion site were performed as previously described (51), except that spectinomycin and streptomycin, each at a concentration of 2.5 µg/ml were used to select for cells where the transposon had transposed into the PCC 7120 genome. To determine average filament lengths, the number of cells in 125 filaments was determined microscopically and averaged. Growth of *Escherichia coli* and concentrations of antibiotics were as previously described (6).

**Plasmid and mutant constructions.** The transposon used in this study was a derivative of Tn1058 constructed by Wolk and coworkers (51). pRL1058, which bears the original transposon on a suicide plasmid, was digested with *MspI* to remove genes conferring resistance to kanamycin, bleomycin, and streptomycin. An  $\Omega$  cassette conferring resistance to streptomycin and spectinomycin was inserted as a *SmaI* fragment from pDW9 (14) to create pRR106, which was used to mutagenize strain PCC 7120.

pAN120 is the suicide plasmid that was used to replace a 592 bp internal fragment of *fraG* with an  $\Omega$  cassette conferring resistance to streptomycin and spectinomycin. A region of DNA corresponding to nucleotides +12 to +817 relative

to the start of *fraG* was amplified using the primers alr2338 UF and alr2338 UR, and a downstream region of *fraG* corresponding to nucleotides +1409 to +2213 was amplified using the primers alr2338 DF and alr2338 DR. The two fragments were cloned into pGEM-T (Promega) and then moved into pHY101, which consists of pBluescript SK+ (Stratagene) with an  $\Omega$  cassette conferring resistance to streptomycin and spectinomycin (14) in the *Hind*III site, on either side of the  $\Omega$  cassette. A fragment consisting of the upstream and downstream regions of *fraG* flanking the  $\Omega$  cassette was moved into pRL278 (5) using *Xho*I and *Spe*I. The *fraG* mutant, UHM127, was created using plasmid pAN120 as previously described (36).

pAN115 is the suicide plasmid that was used to replace a 256 bp internal fragment of PBP6 with an  $\Omega$  cassette conferring resistance to streptomycin and spectinomycin. A region of DNA corresponding to nucleotides +16 to +827 relative to the start of PBP6 was amplified using the primers PBP UF and PBP UR, and a downstream region of PBP6 corresponding to nucleotides +1083 to +1869 was amplified using the primers PBP DF and PBP DR. The two fragments were cloned into pGEM-T (Promega) and then moved into pHY101, on either side of the  $\Omega$  cassette. A fragment consisting of the upstream and downstream regions of PBP6 flanking the  $\Omega$  cassette was moved into pRL278 (5) using *Xho*I and *Spe*I. The PBP6 mutant, UHM128, was created using plasmid pAN120 as previously described (36).

Three GFP transcriptional fusions were made, resulting in plasmids pAN116, which contains nucleotides located -352 to -19 relative to the translational start site of *fraG*, pAN129, which contains nucleotides located -760 to -19 relative to the

translational start site of *fraG* and pAN119 which contains nucleotides located -175 to -15 relative to translational start site of PBP6. Plasmid pAN119 was constructed by amplifying the promoter region of PBP6 using the primers PBP promoter Up and PBP promoter Down. The resultant PCR product was cloned into pGEM-T and subsequently moved into pAM1956 (53) as a *SacI-KpnI* fragment. Plasmid pAN116 was constructed by amplifying the promoter region of *fraG* using the primers alr2338promoterUp and alr2338promoterDown and cloning into pGEM-T. The fragment was then moved to pAM1956 using *SacI* and *KpnI*. For plasmid pAN129 a larger region upstream of *fraG* was amplified using primers UpPalr2338SacIA and Rev 2338. This region was cloned directly into pAM1956 using *SacI* and *KpnI*. Results with plasmids pAN116 and pAN129 were identical. To complement the *fraG* mutant, pAN130 was constructed. Plasmid pAN130 contains promoter region and coding region of *fraG*. This region was amplified using UpPalr2338SacIA and alr2338 end KpnI and was moved into pAM505 (47) using the restriction sites introduced by the primers.

Plasmid pDR138 was used to express *hetR* from the native *hetR* promoter. A 846 bp fragment upstream of the *hetR* coding region was amplified via PCR using primers PhetR-KpnI-F and PhetR-NdeI-R and cloned into pGEM-T.  $P_{hetR}$  was subsequently cloned into pBluescript as an *ApaI-PstI* fragment to create pDR133. *hetR* was amplified from genomic DNA using primers hetRcf-NdeI and hetR6H-r, cloned into pGEM-T and then moved to pDR133 as an *NdeI-PstI* fragment using an *NdeI* site introduced by one of the primers. The subsequent  $P_{hetR}$ -*hetR* fragment was cloned into pAM504 to create pDR138.

### Acetylene reduction, glycolipid and exopolysaccharide assays.

For acetylene reduction assays strains were grown in BG-11 medium to exponential phase. The cultures were then induced by transferring to BG-11<sub>0</sub> and grown under standard conditions for 120 hours. Reduction of acetylene was measured using a gas chromatograph as previously described (6). Thin-layer chromatography and staining of exopolysaccharides were performed as previously described (10, 35).

**Table 1. *Anabaena* strains and plasmids used for studying *fraG* and *pbp6*.**

Strains, plasmids or Oligonucleotides	Characteristic(s)	Source
<i>Anabaena</i> strains		
PCC 7120	Wild type	Pasteur Culture Collection
UHM103	$\Delta$ <i>hetR</i>	(6)
UHM127	<i>fraG</i> mutant	This study
UHM 128	<i>pbp6</i> mutant	This study
Plasmids		
pAN120	Suicide plasmid to inactivate <i>fraG</i>	This study
pAN116	Shuttle vector carrying P <sub><i>fraG</i></sub> - <i>gfp</i> fusion	This study
pAN129	Shuttle vector carrying P <sub><i>fraG</i></sub> - <i>gfp</i> fusion using larger region of DNA upstream of <i>fraG</i>	This study
pAN119	Shuttle vector carrying P <sub><i>pbp6</i></sub> - <i>gfp</i>	This study
pAN115	Suicide plasmid to inactivate <i>pbp6</i>	This study
pSMC127	Shuttle vector carrying P <sub><i>hetR</i></sub> - <i>gfp</i> fusion	(10)
pDR138	Shuttle vector carrying <i>hetR</i> under the control of its native promoter	This study
pPetHetR	Shuttle vector carrying P <sub><i>petE</i></sub> - <i>hetR</i> for copper-inducible expression of <i>hetR</i>	(8)
pAN130	Shuttle vector carrying <i>fraG</i> under the control of its native promoter	This study
pHY101	pBluescript SK+ containing $\Omega$ cassette conferring resistance to streptomycin and spectinomycin	This study
pRR106	Suicide plasmid with transposon, <i>sp</i> <sup>r</sup> , <i>sm</i> <sup>r</sup>	This study
pRL278	Mobilizable suicide vector	(5)

pAM505	Mobilizable shuttle vector	(47)
pAM1956	pAM505 with promoterless <i>gfp</i>	(53)
pGEMT	Cloning vector	Promega
pBluescript SK+	Cloning vector	Stratagene

**Table 2. Oligonucleotides used for studying *fraG* and *pbp6*.**

Oligonucleotides*	Sequence
alr2338 UF	<b>CTCGAGG</b> GAGAAGCGACCAGACAACGACCC
alr2338 UR	<b>ATCGAT</b> GTTACTGGTGTGATTTCTCAGGAG
alr2338 DF	<b>CTGCAGGG</b> TGGTTGTACCACTAATGATAC
alr2338 DR	<b>ACTAGT</b> GCCAATACCAAAAAGCAATTGCC
alr2338promoterUp	<b>GAGCTC</b> GTTGCCAAGTATCCAATTGCAGAAC
UpPalr2338SacIA	AATGGAGCTCGTTCTGAAATATGAGTTATGGCTGG
alr2338promoterDown	<b>GGTACCC</b> ACCTTGACGATACAGCTGCCGC
Rev 2338	<b>CAGTGGT</b> ACCCACCTTGACGATACAGCTGCCGC
<i>fraG</i> end KpnI	<b>TTAAGGT</b> ACCGCCATTATGACTACTGAGCCAGAAG
hetRcf-NdeI	<b>CATATG</b> AGTAACGACATCGATCTGATC
hetR6H-r	TTAGTGATGGT <b>GATGGT</b> GATGATCTTCTTTCTACCAA ACACCATTG
PhetR-KpnI-F	<b>GGTACCC</b> CTGCCAATGCAGAAGGTTAAAC
PhetR-NdeI-R	<b>CATATG</b> ACAAATAGTTGAATAGCAGCGTTATTAG
PBP UF	<b>CTCGAG</b> GCAACCGCAACGTCGTA <b>CTTCGTC</b>
PBP UR	<b>ATCGAT</b> GAATGTAAGTTCTT <b>CCTTGAGAGC</b>
PBP DF	<b>CTGCAG</b> CTAGTCGGTGGTGTAGACTCTAG
PBP DR	<b>ACTAGT</b> GCACGCCCTTGAGTGCCTTAGTC
PBP promoter Up	<b>GAGCTC</b> CTTTAAGAGATTCGCTATTA <b>ACTACG</b>
PBP promoter Down	<b>GGTACCC</b> CTCACTTGTA <b>AAATATTACTGTAATTC</b>
PBP FI Re For	<b>GTGAAAT</b> CGCCGTGTCGTCTAGGAC
PBP FI Re Rev	<b>CCAGTCC</b> CTTAATTCGCCTTAGGACG

\*Oligonucleotides read in the 5' to 3' direction. Nucleotides in bold print indicate a restriction site engineered into the sequence.

## RESULTS

***pbp6* is essential for diazotrophic growth.** Transposon mutagenesis yielded a mutant unable to grow under diazotrophic conditions that was interrupted by the transposon in the coding region of PBP6. The protein PBP6 is one among twelve PBPs in *Anabaena* (29). In order to confirm that the insertion of the transposon in the coding region of PBP6 is the real cause for the phenotype, the mutation was recreated by replacing internal fragment of the gene by an  $\Omega$  cassette to obtain the strain UHM128.

The mutant strain UHM128 has elongated vegetative cells in the presence and absence of nitrate. The heterocysts produced in nitrate free media are also elongated. The cyanophycin granules are not present at the poles. Instead, the heterocysts seem to be opened at the poles. The junction between the heterocysts and the vegetative cells tend to break easily. Hence the filaments are short in nitrate-depleted media (Figure 1). Heterocysts are detached from the filaments and are clustered together. The growth rate of UHM128 was determined in media supplemented with nitrate and without nitrate, to find out whether the growth rate was affected by the inactivation of the gene *pbp6*. The mutant strain UHM128 did not show any significant difference in growth rate when compared to the wild type in media supplemented with nitrate (Figure 2). However, UHM128 failed to grow in nitrate free media (Figure 3).

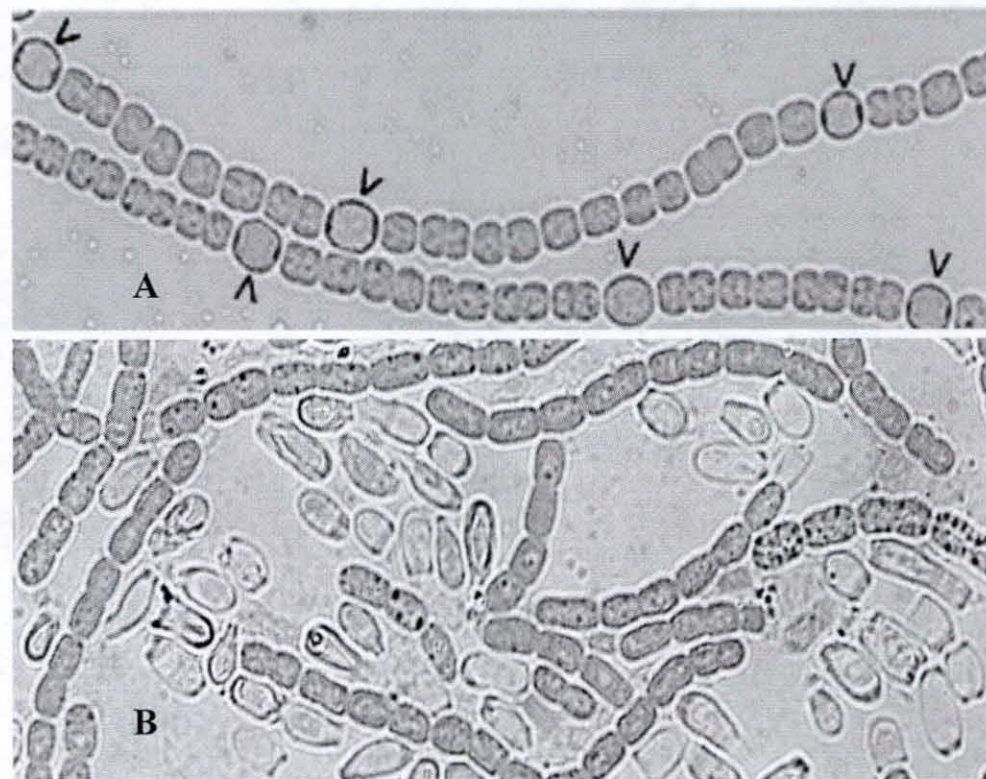


Figure 1. Phenotype of UHM128. The mutant has elongated cells, heterocysts seem to be opened at one end and filaments are shorter when compared to that of PCC7120. The wild-type strain PCC 7120 24 h postinduction (A). Strain UHM128 24 h postinduction (B).

### Growth Rate of UHM128 in BG-11

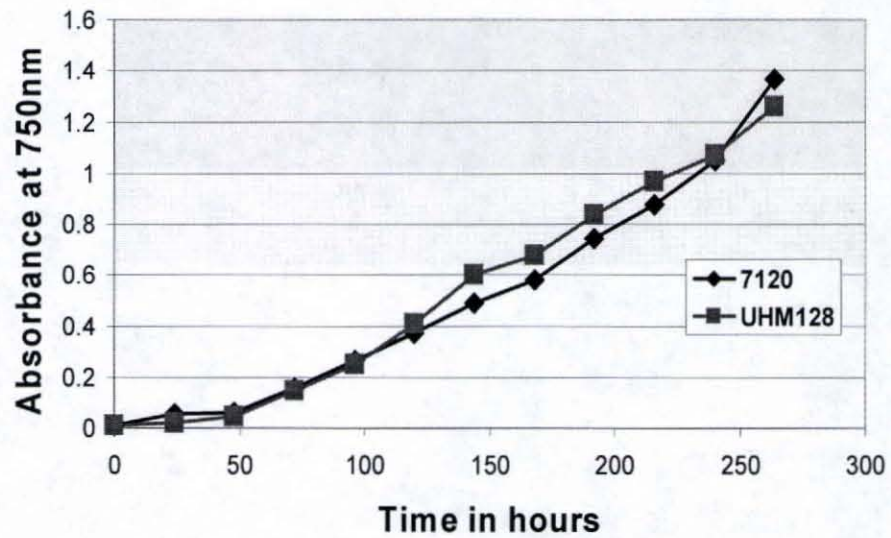


Figure 2. Growth rate of UHM128 is similar to that of wild-type in BG-11

### Growth Rate of UHM128 in BG-11<sub>0</sub>

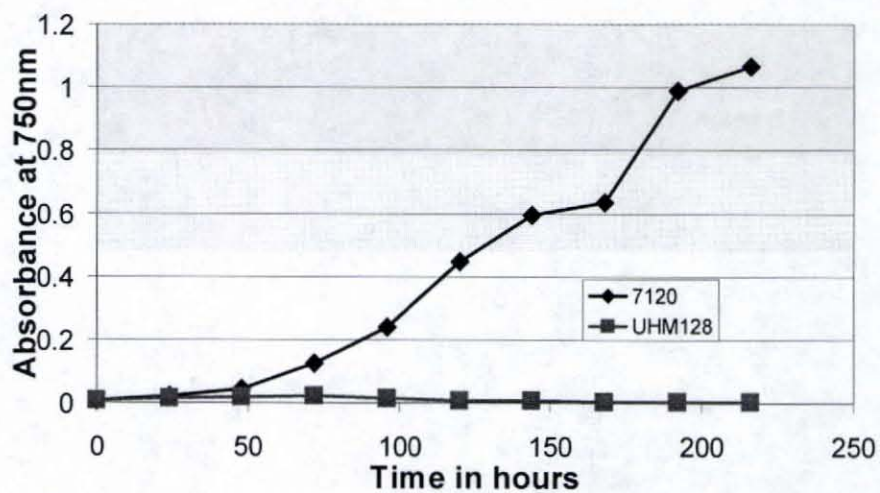


Figure 3. UHM128 is unable to grow in BG-11<sub>0</sub>

## Nitrogenase activity of UHM128

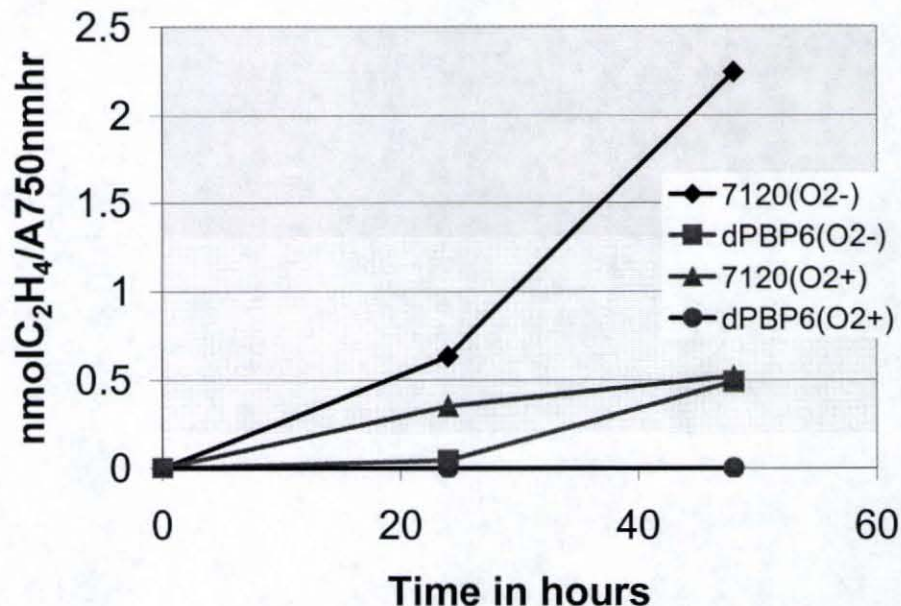


Figure 4. UHM128 is a  $fox^-$  mutant

PBPs in *E. coli* are involved in the synthesis of peptidoglycan layer. Since the growth rate of UHM128 was affected in a medium lacking nitrate, it was speculated that the defective peptidoglycan layer in UHM128 might be allowing the entry of atmospheric oxygen. And the oxygen that enters the cells possibly could inactivate the oxygen labile nitrogenase. Hence, acetylene reduction assays were used to assess nitrogenase activity. UHM128 did not show any nitrogenase activity under aerobic conditions. However, acetylene reduction assays performed under anaerobic conditions, showed that UHM128 was able to fix atmospheric nitrogen in the absence of oxygen (Figure 4).

**Expression of *pbp6* is induced in both vegetative cells as well as heterocysts.** To determine the timing and location of the expression of *pbp6*, a  $P_{pbp6}$ -*gfp* fusion was made and introduced into the wild type strain on a shuttle vector. All cells, vegetative cells as well as heterocysts, had a uniform and high level of fluorescence indicating transcriptional activity from the promoter in media with and without nitrate. The constitutive and high promoter activity in both vegetative cells and heterocysts reinforces the fact that the peptidoglycan layer is a common feature in both vegetative cells and heterocysts.

While this work was proceeding in our lab, Leganes *et. al.* (29) published similar results on PBP6. Hence, this experiment was not pursued further.

### ***fraG* is necessary for diazotrophic growth and filament integrity.**

In a screen for mutants that could not grow in the absence of a source of fixed nitrogen, a transposon used to increase the rate of mutagenesis and mark the site of the mutation had inserted into the same gene in four of the mutants isolated. The gene designated *alr2338* during annotation of the genome (24), which we have named *fraG* (fragmentation mutant that lacks heterocysts specific glycolipids), was interrupted at nucleotide positions +124, +158, +176, and +1770 relative to the predicted translational start site. To confirm a cause-and-effect relationship between the insertions and the mutants' inability to grow diazotrophically an internal region of *fraG* in the wild type strain, PCC 7120, was replaced by an  $\Omega$  cassette. The resulting strain, UHM127, was unable to grow diazotrophically. Because *fraG* is located

immediately upstream of *hetR*, the master regulator for heterocyst differentiation, *hetR* and its promoter region were introduced into the mutant on a shuttle vector to see if the phenotype was the result of a polar effect on *hetR*. The wild-type phenotype was not restored to the mutant by the addition of *hetR*, whereas addition of a wild-type copy of *fraG* restored the wild-type phenotype, indicating that the phenotype of UHM127 was caused by inactivation of *fraG*.

The gene *fraG* encodes a putative protein of 751 amino acids. One complete and one partial DUF6 domain were detected at the carboxy-terminal end of the protein. DUF6 domains are indicative of integral membrane proteins, and it is common for proteins to contain two. The carboxy-terminal half of the protein is predicted to contain 10  $\alpha$ -helical transmembrane domains (30).

The rate of growth of the mutant strain, UHM127, was similar to that of the wild type in media containing nitrate as a fixed nitrogen source. However, the average filament length was substantially reduced to about 50 cells, compared to more than 200 for the wild type. Unlike the wild type, which forms a pattern of single heterocysts along the filament (Figure 5A), UHM127 fragmented when deprived of fixed nitrogen. Fragmentation started between 16 and 18 hours after nitrogen deprivation, and at 24 hours, single cells and filaments with an average filament length of about 9 cells were present together (Figure 5B) compared to only intact filaments with an average length of 150 cells for the wild type. The single cells in the mutant culture were less pigmented than the cells in the filaments, reminiscent of the reduced pigmentation in heterocysts caused by the degradation of phycobilisomes. After 48 hours of nitrogen deprivation the average number of cells for the mutant was

5 and for the wild type it was 145. At 72 hours and thereafter, the mutant culture was mostly unicellular (Figure 5C). No mature heterocysts were observed in the mutant culture at any time.

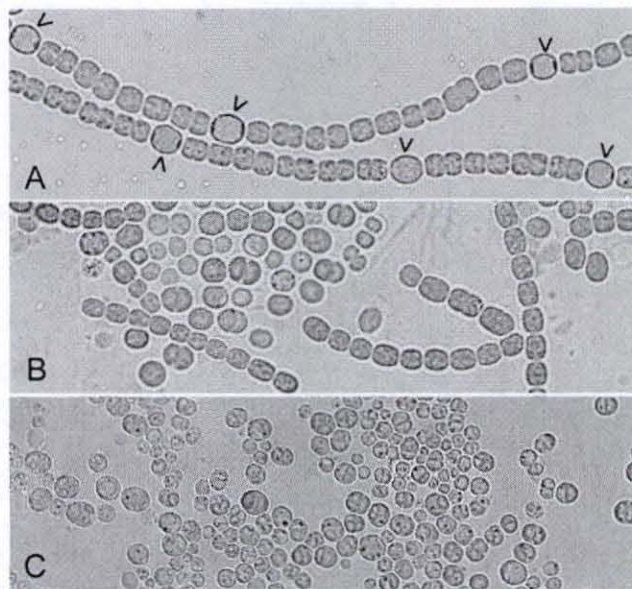


Figure 5. Phenotype of UHM127. UHM127 in BG-11<sub>0</sub> forms shorter filaments and eventually the mutant culture becomes unicellular. No mature heterocysts are seen. The wild-type strain PCC 7120 24 h postinduction (A). Strain UHM127 24 h postinduction (B). Strain UHM127 72 h postinduction (C).

To determine if UHM127 was a  $\text{Fix}^-$  (unable to fix in the presence of molecular oxygen) or  $\text{Fix}^-$  (unable to fix under any conditions) mutant, acetylene reduction assays were performed. The wild type reduced acetylene in the presence and absence of oxygen, a *pbp6* mutant, which is known to have a  $\text{Fix}^-$  phenotype (29), was used as a control for anaerobic conditions and could only fix in the absence

of oxygen as expected, and UHM127 could not fix under either condition (Data not shown). UHM127 was therefore categorized as a  $\text{Fix}^-$  mutant with developmental defects.

**Fragmentation can be elicited by differentiation alone.** Deprivation of a fixed source of nitrogen induces a developmental program in *Anabaena* sp. PCC 7120 that culminates in the formation of a pattern of heterocysts along a filament. To examine whether deprivation of fixed nitrogen or induction of differentiation was the more direct cause of fragmentation of the mutant strain UHM127, differentiation was induced in media containing fixed nitrogen and the phenotype of the mutant was observed. Extra copies of *hetR* under the control of the copper-inducible *petE* promoter on plasmid pPetHetR cause differentiation of heterocysts in the wild-type strain in the presence of nitrate or ammonia when copper is present in the medium (8). UHM127 carrying pPetHetR with nitrate in the absence of copper had filaments similar to the same strain without the plasmid. However, when copper was included in the medium to induce expression of *hetR* and heterocyst formation, the strain fragmented in a manner similar to the mutant strain in a medium lacking fixed nitrogen (Figure 6). Fragmentation in the presence of fixed nitrogen when differentiation is induced by overexpression of *hetR* suggests that differentiation, and not a lack of fixed nitrogen in the medium, was the more direct cause of fragmentation by the mutant.

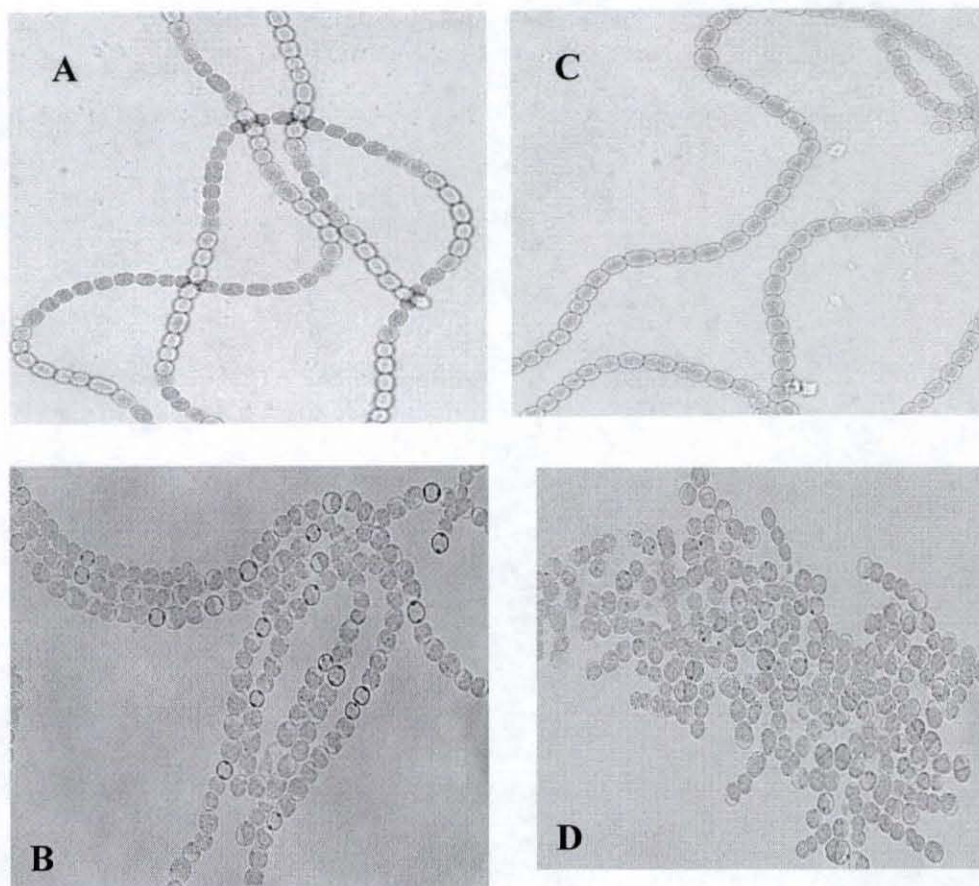


Figure 6. Fragmentation can be elicited by differentiation. The strain PCC 7120 on BG-11 medium without copper (A). The strain PCC 7120 with the plasmid pPetHetR on BG-11 medium supplemented with copper (B). UHM127 on BG-11 medium without copper (C). UHM127 with the plasmid pPetHetR on BG-11 medium supplemented with copper (D).

***fraG* is not necessary for pattern formation.** The absence of mature heterocysts in strain UHM127 indicated that *fraG* was necessary for complete differentiation of heterocysts. To determine if *fraG* is also necessary for determining which cells will differentiate, a *hetR-gfp* transcriptional fusion on plasmid pSMC127 (10) was introduced on a shuttle vector into both the mutant and the wild type. In the wild type, a pattern of expression of *hetR* in single cells that predicts the pattern of cells that differentiate into heterocysts can be visualized with the GFP fusion between approximately 8 - 24 h after removal of combined nitrogen. In UHM127 a pattern of GFP fluorescence was seen in proheterocysts 12 hours after induction before fragmentation starts in the mutant (Figure 7). A pattern of induction of the *hetR* promoter in single cells separated by approximately 10 cells with lower fluorescence, similar to that in the wild type, was seen in the mutant, suggesting that *fraG* is not necessary for proper pattern formation.

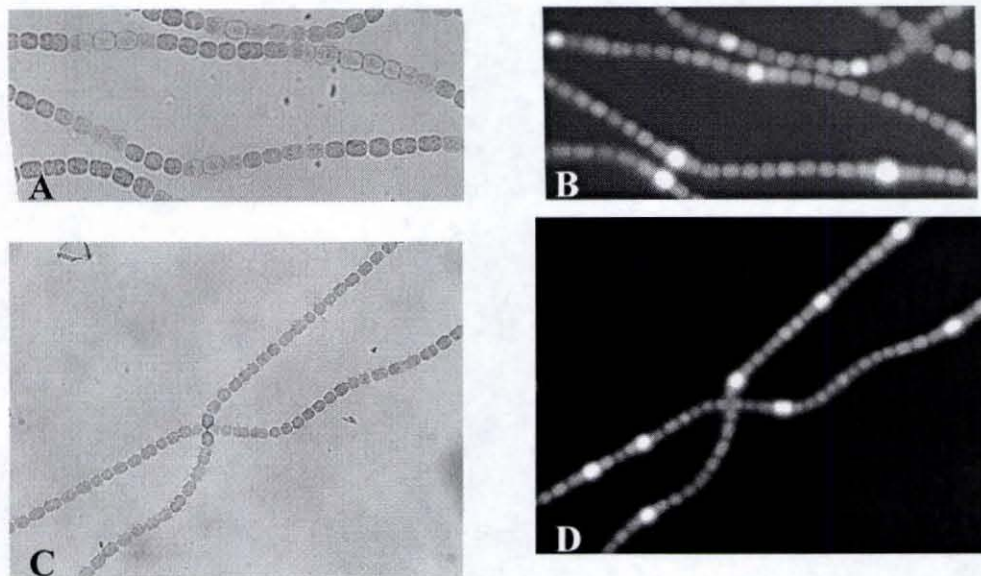


Figure 7: Expression of  $P_{hetR}$  in UHM127. UHM127 can initiate normal pattern formation upon deprivation of fixed source of nitrogen. The strain PCC 7120 with the plasmid pSMC127 under visible light (A) and under ultra violet light (B). UHM127 with the plasmid pSMC127 under visible light (C) and ultra violet light (D).

**Expression of *fraG* is induced in heterocysts.** To determine the temporal and spatial aspects of expression of *fraG* under different culture conditions, the promoter region of *fraG* was fused to *gfp*, and the construct was introduced into the wild-type strain on a shuttle vector. With nitrate in the medium a low level of fluorescence was observed in all vegetative cells of filaments for *fraG*. The level of fluorescence was significantly higher than that of the same strain carrying a promoterless version of the construct, indicating that levels of expression from the

*fraG* promoter was at a low, uniform level along the filament in the presence of nitrate. In contrast, in filaments with heterocysts, there was a moderate increase in fluorescence from mature heterocysts compared to that from intervening vegetative cells, which retained the level of fluorescence seen in the presence of nitrate (Figure 8). From the time of removal of fixed nitrogen from the culture to the appearance of mature heterocysts, the same low, uniform level of fluorescence was observed as from filaments grown in media containing nitrate. Therefore, transcription of *fraG* appears to be induced slightly in heterocysts, or perhaps in proheterocysts just prior to maturation

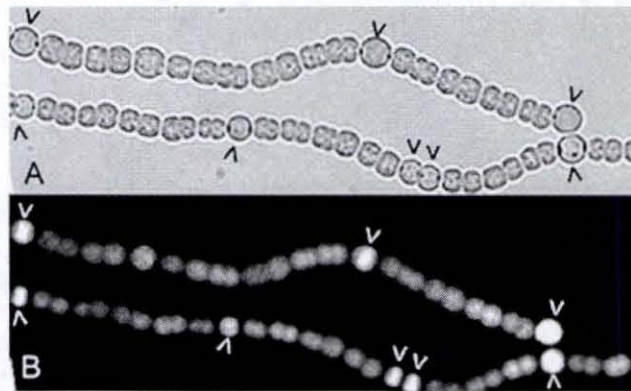


Figure 8. Expression of *fraG* is moderately increased in heterocysts. PCC 7120 carrying a  $P_{fraG}$ -*gfp* transcriptional fusion on plasmid pAN116 48 h after induction of heterocyst formation under visual light (A) and ultraviolet light (B).

### ***fraG* is necessary for synthesis of heterocyst glycolipids.**

Although cells with larger size and thickened cell envelopes, two of the attributes of mature heterocysts, were not seen in the *fraG* mutant, single cells with reduced pigmentation appeared to break off and fragment filaments between 16 and 18 h post

induction. These cells appeared to be proheterocysts that were arrested at a certain stage of development. To determine if heterocyst-specific exopolysaccharides and glycolipids, which are involved in the late stages of heterocyst maturation and necessary for creation of a microaerophilic environment in functioning heterocysts, were produced in the *fraG* mutant, UHM127 was checked for their presence. Alcian Blue, which binds specifically to heterocyst envelope polysaccharides, stained the cells with reduced pigmentation that broke from the filaments between 16 and 18 h postinduction, confirming that they were proheterocysts and indicating that *fraG* was not necessary for exopolysaccharide synthesis or deposition. At 15 h postinduction prior to fragmentation, a pattern of alcian blue stained cells was visible along filaments (Data not shown).

Glycolipids from both the wild-type strain and the mutant were separated by thin layer chromatography and visualized. Samples of the wild type harvested at 48 h postinduction contained the two heterocyst-specific glycolipids, whereas those from UHM127 did not, indicating that *fraG* was necessary for their production (Figure 9). UHM127 complemented with a wild-type copy of *fraG* on a plasmid produced similar types and quantities of glycolipids as the wild type. The absence of heterocyst glycolipids in the mutant implied that either *fraG* performs a function that may be directly required for glycolipid synthesis or, alternatively, fragmentation arrested development before glycolipid synthesis begins in the wild type and their absence in the mutant was an indirect consequence of mutation of *fraG*. To distinguish between these two possibilities, glycolipids from the mutant and the wild type were visualized at 15 h postinduction, before fragmentation of the mutant. One of the two heterocyst

glycolipids was clearly visible in samples from the wild type 15 h postinduction, indicating that glycolipid synthesis begins before the time of fragmentation of the UHM127 (Figure 9). The mutant, however, had not produced heterocyst glycolipid at this time when filaments were still intact, indicating that fragmentation most likely did not arrest development prior to synthesis of glycolipid to stop its production. Instead, it appears that *fraG* has a function necessary for glycolipid synthesis in addition to maintaining filament integrity during differentiation.

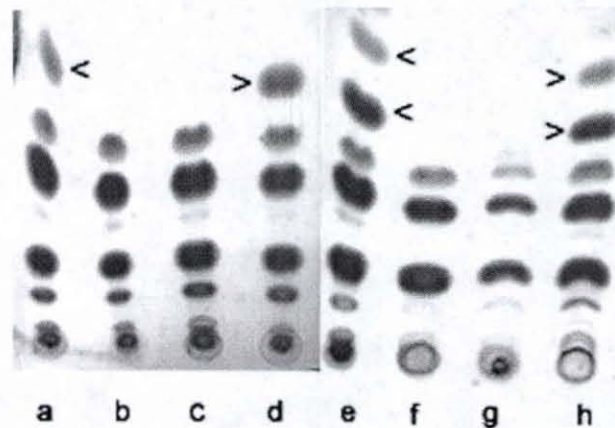


Figure 9. Thin layer chromatography of glycolipids produced by the wild-type strain PCC 7120, the  $\Delta$ *hetR* mutant UHM103, and the *fraG* mutant UHM127. UHM127 is not able to produce heterocysts specific glycolipids. Lanes a – d represent samples collected at 15 h postinduction. Lanes e – f represent samples collected at 48 h postinduction. Lanes a and e, PCC 7120; lanes b and f, UHM103; lanes c and g, UHM127; and lanes d and h, UHM127 complemented with plasmid pAN130. Carets indicate heterocyst glycolipids

## DISCUSSION

A handful of genes have been described that are necessary for filament integrity in *Anabaena* sp. PCC 7120 and cause an increased fragmentation phenotype under diazotrophic conditions. Buikema and Haselkorn described the isolation of 4 mutants with Fox<sup>-</sup>, fragmentation phenotypes in a study that also led to the identification of *hetR* (9). For one mutant, the mutation necessary for the phenotype was found to disrupt the *fraC* gene, which encodes a phenylalanine-rich peptide with 4 potential transmembrane domains (2). In contrast to the *fraG* mutant, UHM127, described here, a *fraC* mutant can fix nitrogen in the absence of molecular oxygen, mature heterocysts are occasionally observed and can be induced by introducing extra copies of *hetR* on a plasmid, and the fragmentation phenotype is more severe in the presence of fixed nitrogen. Similarly, mutation of *fraH*, which encodes a proline-rich peptide, causes fragmentation of filaments, but mature heterocysts are common in the mutant. The genetically uncharacterized fragmentation mutant N5 described by Wolk and coworkers, on the other hand, shows no signs of differentiation (12) unlike UHM127, which develops a normal pattern of proheterocysts. Finally, Golden and coworkers inactivated some group 2 sigma factors from *Anabaena* sp. PCC 7120 and found that a *sigE*, *sigD* double mutant fragmented upon removal of fixed nitrogen, but mature heterocysts were formed (25), suggesting that these two sigma factors are more likely involved in transcription of *fraC* or *fraH* than that of *fraG*.

The increased fragility of the majority of fragmentation mutants in both nitrogen-replete and nitrogen-free media suggests that the integrity of heterocyst-vegetative cell junctions may rely on several proteins that are also necessary for

vegetative cell junctions, rather than being dependent on a separate set of proteins specific to heterocyst junctions. A low level of expression of *fraG* in vegetative cells followed by induction in heterocysts is consistent with this idea.

The somewhat pleiotropic effect of mutation of *fraG*, which resulted in Fix<sup>-</sup>, Hgl<sup>-</sup>, Fra phenotypes, suggests *fraG* affects both structural and regulatory aspects of heterocyst development, with a defect in one affecting the other. As an integral membrane protein, it may have primarily a structural role that is necessary for the advancement beyond a certain point in the developmental program. In this case, regulation of differentiation would involve sensing of membrane or cell-junction structure. The formation of heterocyst-specific glycolipid by the wild type at a time before fragmentation of the mutant coupled with the absence of glycolipid synthesis in the mutant suggests that fragmentation is not the structural change that prevents differentiation in the absence of *fraG*. Conversely, the primary function of *fraG* may be in transport or signal transduction that is necessary for development, and disruption of development initiates a genetically programmed response that leads to fragmentation. Fragmentation of the wild-type strain under some environmental conditions and after the death of heterocysts when filaments are returned to nitrogen-replete conditions may confer a selective advantage under certain conditions, presumably by facilitating cell dispersion.

## CHAPTER 3

### **Transcription of *hfq* is induced in proheterocysts and mature heterocysts of *Anabaena* sp. PCC 7120**

Hfq (host factor protein q) is a small, abundant, autoregulated and thermostable hexamer in *Escherichia coli* (44). It was first isolated as a bacterial host factor essential for *Escherichia coli* phage Q $\beta$  replication (16). Hfq belongs to the family of Sm-like proteins that are involved in nuclear splicing in eukaryotes (32, 55). Recent evidence shows the involvement of Hfq in regulation of translation by its own and/or by combining with a sRNA and in degradation of mRNA.

Hfq acts as a polyfunctional translational regulator for many RNAs. The effect of Hfq can be either direct or indirect. Hfq, a RNA chaperone, has been shown to bind to the target mRNAs and mediate the interaction between the mRNA and a corresponding sRNA. It affects the stability of many mRNAs by binding to A/U rich regions (38) and targeting them for degradation by increasing polyadenylation. It can interfere with the ribosomal binding and thus with translation. Hfq activates the translation of the mRNA for the bacterial RNA polymerase  $\sigma^s$  subunit, which is a global regulator (33). A mutation in the *hfq* gene suppresses or activates synthesis of more than 50 proteins in *E.coli* (33). The deletion of *hfq* in *E.coli* produces a pleiotropic phenotype which includes lower negative supercoiling of plasmids in stationary phase, higher osmosensitivity, higher sensitivity to oxidants and UV irradiation, lower growth rate, lower cell number in culture and rapid carbohydrate oxidation (43).

In nitrogen fixing bacteria, evidence suggests that Hfq has a significant role in nitrogen fixation. In *Azorhizobium caulinodans*, a symbiotic nitrogen fixing organism in *Sesbania rostrata* (legume), NrfA (*nif* regulatory factor) is functionally equivalent to *E. coli* Hfq. NrfA is a RNA-binding like protein homologous to *E. coli* Hfq. It was able to restore the defect of *rpoS* (*rpoS* encodes sigma factor for RNA polymerase) translation in an *hfq* mutant of *E. coli* (23). NifA, a transcriptional activator, is required for the expression of *nif* genes and *fix* genes (1, 37). A NrfA mutant in *Azorhizobium caulinodans* has a pleiotropic phenotype, which is Nod<sup>+</sup> Nif<sup>-</sup>, Fix<sup>-</sup>. At least 10 to 15 polypeptides synthesized under conditions of nitrogen fixation were not detectable. There are two mechanisms by which NrfA is thought to affect the expression of *nifA*. NrfA is believed to contribute to *nifA* mRNA stability and/or translation. The mRNA of NifA has a leader sequence to which NrfA can bind and protect from degradation. In the absence of NrfA, this is susceptible to degradation by nucleases (23). In *E. coli*, Hfq binds to supercoiled and linear DNA non-specifically along with 11 other proteins. Thus, Hfq in *E. coli* is involved in nucleoid formation (39, 40). DNA topology changes when there is a shift from aerobiosis to anaerobiosis. NrfA might be involved in this change, thus affecting *nifA* transcription *via* an effect on chromosome structure. In *Rhodobacter capsulatus*, NrfA is not essential for growth under nitrogen-repleted conditions. It is required for maximal synthesis of nitrogenase and thus in maximal diazotrophic growth (11).

Since *nrfA*, a homologue to *E. coli hfq* (host factor protein q), is essential for diazotrophic growth in *A. caulinodans* and for maximal synthesis of nitrogenase in *R. capsulatus*, a reverse genetic analysis was attempted to examine the role of *hfq* in

nitrogen fixation in *Anabaena*. In *Anabaena*, the *asl2047* (*hfq*) gene predicts a protein of 72 amino acids with a molecular weight of 7900 Da and has a putative conserved domain recognized by NCBI, which is 88.5% similar to that of *hfq* (Figure 10.)

The gene is 97% similar to that of *Anabaena variabilis* ATCC 29413 and 75% similar to that of *Nostoc punctiforme* PCC 73102.

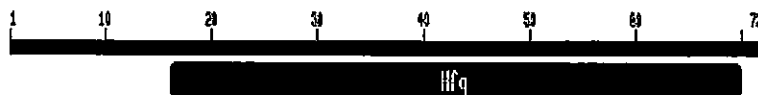


Figure 10. Putative conserved domain of Hfq recognized by NCBI

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Refer to materials and methods in Chapter 2

**Strain and plasmid constructions.** Table 1 lists the *Anabaena* strains, plasmids and oligonucleotides used in this study. pAN105 is the suicide plasmid that was used to replace 325 bp containing the coding region of *hfq* with an  $\Omega$  cassette conferring resistance to streptomycin and spectinomycin. A region of DNA corresponding to nucleotides -752 to -14 relative to the translational start site of *hfq* was amplified using the primers hfq UF and hfq UR HindII, and a downstream region of *hfq* corresponding to nucleotides +311 to +914 was amplified using the primers Hfq DF and Hfq DR. The two fragments were cloned into pGEM-T (Promega) and then moved into pHY101, which consists of pBluescript SK+ (Stratagene) with an  $\Omega$  cassette conferring resistance to streptomycin and spectinomycin (14) in the *HindIII* site, on either side of the  $\Omega$  cassette. A fragment consisting of the upstream and downstream regions of *hfq* flanking the  $\Omega$  cassette was moved into pRL278 (5) using *XhoI* and *SpeI*. The  $\Delta hfq$  mutant, UHM129, was created using plasmid pAN105 as previously described (36).

Plasmid pMUB100 is a shuttle vector containing  $P_{hfq-gfp}$  used for the transcriptional fusion. A region from -14 to -228 bp upstream of the start codon of *hfq* was presumed to contain the promoter region of *hfq*. This region was amplified using the primers Promo Hfq forward and Promo Hfq reverse using PCR and cloned into the pGEM-T and subsequently transferred to pAM1956 using *SacI* and *KpnI*.

Plasmid pAN131 is a shuttle vector containing *hfq-gfp* translational fusion. A region from -228 to +116 bp with respect to the translational start site of *hfq* was amplified *via* PCR using the primers Hfq Translational fusion F SacI and Hfq Translational fusion R Linker. GFP was amplified using the primers GFP Translational fusion F Linker and GFP-R KpnI. The two PCR fragments were fused together by overlap extension PCR as previously described (19). The resultant PCR product was moved into pAM505 as *SacI* and *KpnI* fragment.

**Table 3. *Anabaena* strains and plasmids used for studying *hfq***

Strains, plasmids or Oligonucleotides	Characteristic(s)	Source
<i>Anabaena</i> strains PCC 7120	Wild type	Pasteur culture collection
UHM 103	$\Delta$ <i>hetR</i>	(6)
UHM 129	$\Delta$ <i>hfq</i>	This study
pAN105	Suicide plasmid to delete <i>hfq</i>	This study
pMU100	Shuttle vector carrying $P_{hfq}$ - <i>gfp</i>	This study
PAN131	Shuttle vector carrying <i>hfq-gfp</i>	This study
pAM1956	pAM505 with <i>gfp</i> , mobilizable shuttle vector	(53)
pGEM-T	Cloning vector	Promega
pHY101	pBluescript SK+ containing $\Omega$ cassette conferring resistance to streptomycin and spectinomycin	This study
pRL278	Mobilizable suicide vector	(5)

**Table 4. Oligonucleotides used for studying *hfq***

Oligonucleotides	Sequence
hfq UF	<b>CTCGAGGTCACGGCTGACAACTTGAATAAATTC</b>
hfq UR HindII	<b>GTCAACGGAATAATTGAGGGATTAAGTCATAGG</b>
hfq DF	<b>CTGCAGCTTTTAGCGTCTCGTAGAGAGCGTC</b>
hfq DR	<b>ACTAGTGAAGAAATCGATTATCCCAGCAATC</b>
Promo Hfq forward	<b>GAGCTC CTTTCTCTGGAGTGATTGCCGGCG</b>
Promo Hfq reverse	<b>GGTACC GAATAATTGAGGGATTAAGTCATAGG</b>
Hfq Fl.Reg. Forward	<b>GTAATTCCTGCACCACGTTCCCAAAC</b>
Hfq Fl.Reg. Reverse	<b>CAGGCGTAAGCTTAATGGCAATCC</b>
Hfq Translational fusion F SacI	<b>CTCTGAGCTCCTTTCTCTGGAGTGATTGCCGGCG</b>
Hfq Translational fusion R Linker	<b>ACTTTTACGACTTCTTGCACTCCTACCTGTAATTGCATCTCCTGTC</b>
GFP Translational fusion F Linker	<b>AGTGCAAGAAGTCGTA<del>AAA</del>AGTATGAGTAAAGAGAAGAACTTTTCAC</b>
GFP-R KpnI	<b>GCTAGGTACCTTATTTGTATAGTTCATCCATGCC</b>

\*Oligonucleotides read in the 5' to 3' direction. Nucleotides in bold print indicate a restriction site engineered into the sequence

**Growth rate determination.** For growth rate determination, a culture that had reached the  $A_{750}$  of 0.6 was used for inoculating the second culture. The culture was inoculated to an absorbance of 0.05 for growth rate determination in the presence of nitrate and with an absorbance of 0.07 for that in the absence of nitrate.

**Acetylene Reduction Assay.** The strains were grown in BG-11 medium to exponential phase and induced by transferring to BG-11<sub>0</sub> followed by growth under standard conditions for 120 hours. Reduction of acetylene was measured using a gas chromatograph as previously described (2)

**Southern Blot and PCR.** Southern Blot was performed to confirm the insertion of  $\Omega$  cassette in the coding region of *hfq*. Chromosomal DNA was extracted from two isolates of the UHM129 strains (UHM129 isolate # 1 and UHM129 isolate # 3) as well as from the wild type. Biotinylated probe was made with NEBlot Phototope Kit (New England Biolabs). *PfoI* and *BstZ171* were enzymes used to digest chromosomal DNA of UHM129 strains and the wild type. The 627 fragment downstream of *hfq* used in the construction of pAN105 was used as a probe. *PfoI* digested the chromosomal DNA on either side of the *hfq* gene and a predicted band size of 6 kb was obtained in the wild type and a predicted band size of 8 kb was obtained in UHM 129 since the  $\Omega$  cassette (2 kb) was inserted. Similarly upon the digestion of the chromosomal DNA with *BstZ171*, a predicted band size of 2.5 kb was obtained and a predicted band size of 4.5 kb was obtained with UHM 129.

PCR was performed to confirm the presence of  $\Omega$  cassette in the coding region of *hfq* in UHM 129. Chromosomal DNA of UHM129 and the wild type were subjected to PCR using two primers (Hfq Fl.Reg. Forward and Hfq Fl.Reg. Reverse) outside the flanking regions used for cloning. In UHM129, since the  $\Omega$  cassette was inserted, the expected band size was more than that in the wild type. For the

UHM129, the size of the PCR fragment obtained was about 3.5 kb and for the wild type it was about 1.5 kb, as expected.

## RESULTS

**Hfq is not necessary for diazotrophic growth.** Since Hfq is an essential protein in many prokaryotes, a reverse genetic approach was taken to study about the protein in *Anabaena*. As the first step, UHM129 was created by replacing the coding region of *hfq* by an  $\Omega$  cassette. Gene inactivation was confirmed by PCR (Figure 11) and southern blot (Figure 12).

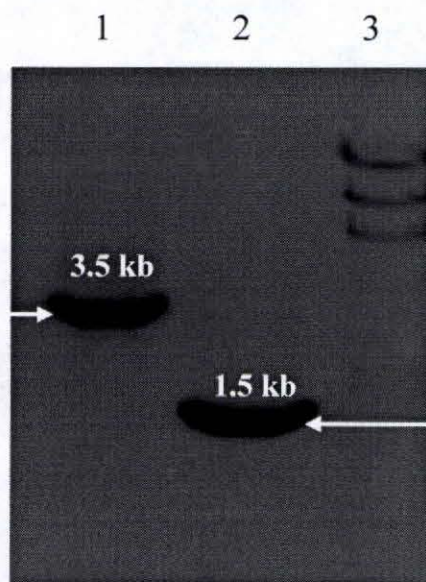


Figure 11. PCR for confirming the insertion of  $\Omega$  cassette in the coding region of *hfq*. Lane 1 represents UHM129. Lane 2 represents the wild type strain PCC 7120. Lane 3 represents the molecular marker  $\lambda$  DNA cut with *Hind*III.

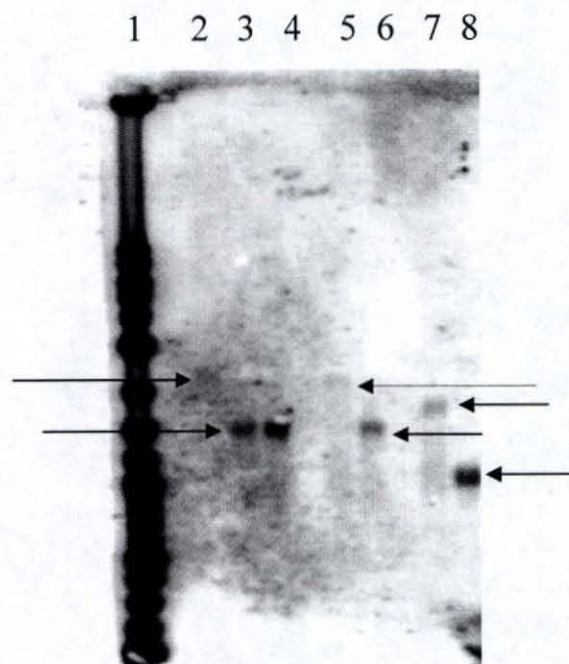


Figure 12. Southern Blot for confirming the insertion of  $\Omega$  cassette in the coding region of *hfq*. Lane 1 represents molecular weight marker. Lane 2 represents UHM129 isolate # 1 digested with *PfoI* (about 8 kb). Lanes 3 and 4 represent UHM129 isolate # 1 digested with *BstZ171* (about 4.5 kb). Lane 5 represents UHM129 isolate # 3 digested with *PfoI* (about 8 kb). Lane 6 represents UHM129 isolate # 3 digested with *BstZ171* (about 4.5 kb). Lane 7 represents PCC 7120 digested with *PfoI* (about 6 kb). Lane 8 represents PCC 7120 digested with *BstZ171* (about 2.5 kb)

The phenotype of UHM129 was determined by microscopic observation. It appeared to be similar to the wild type in conditions with and without nitrate. The percentage of heterocysts also seemed to be similar to that of the wild type (Figure 13)

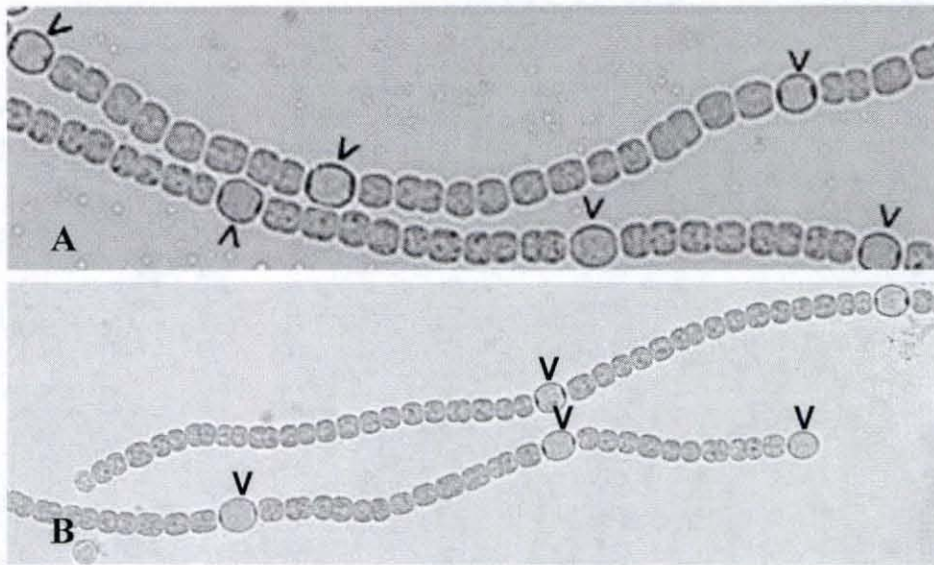
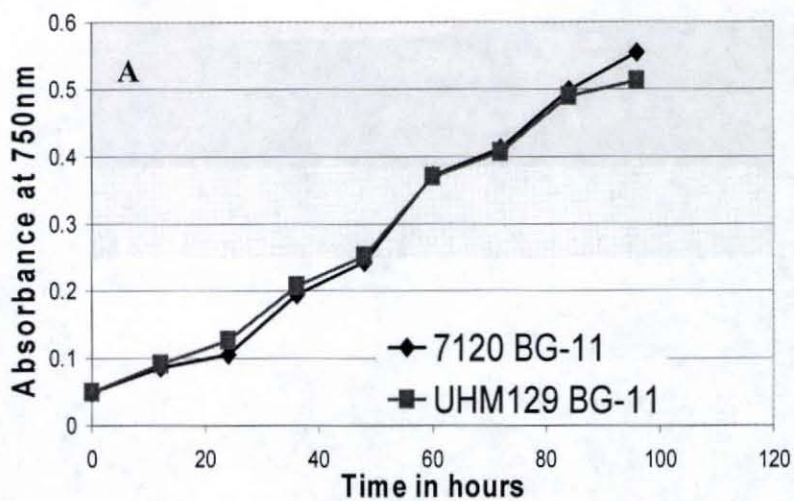


Figure 13. Phenotype of UHM129 is similar to that of PCC7120. The wild-type strain PCC 7120 24 h postinduction (A). Strain UHM129 24 h postinduction (B).

The growth rate of UHM129 was determined in a medium supplemented with nitrate and in a medium without nitrate. There was no significant difference between the growth rate of the mutant and wild type (Figure 14).

### Growth Rate of UHM129 in BG-11



### Growth Rate of UHM129 in BG-11<sub>0</sub>

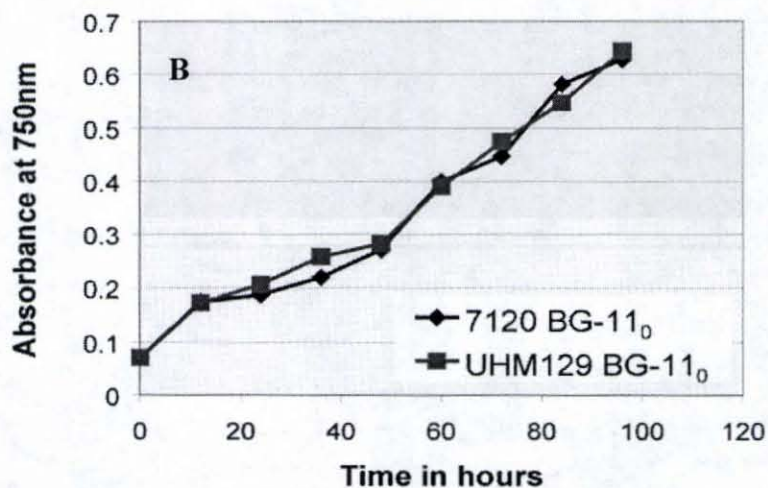


Figure 14. Growth rates of UHM129 and PCC 7120 are similar in both BG-11 and BG-11<sub>0</sub>. Growth rate determination in the presence (A) and absence (B) of nitrate.

An acetylene reduction assay was done to determine the nitrogenase activity in UHM129. There was no significant difference in nitrogen fixation between the mutant and the wild type (Figure 15).

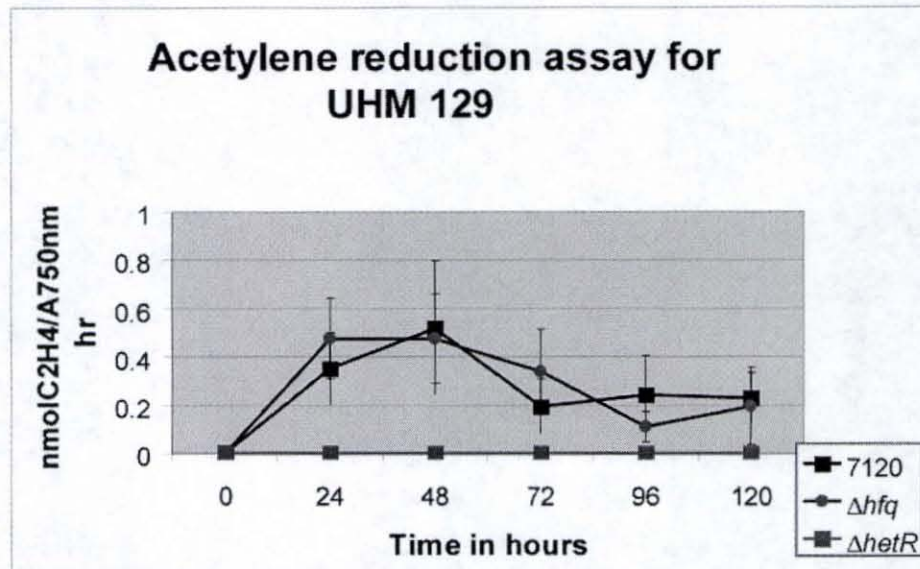


Figure 15. Nitrogenase activity of UHM129 is similar to that of wild-type

**Expression of *hfq* is induced in proheterocysts and mature heterocysts.** Transcriptional fusion helps to study promoter activity as well as mRNA stability of the gene *hfq*. Location, timing and the intensity of gene expression were determined. The promoter region of *hfq* was cloned into pAM1956 and the resultant shuttle vector was introduced into UHM129 and the wild type. In the presence of a fixed source of nitrogen, there was a uniform level of expression in all vegetative cells. However, in the absence of a fixed source of nitrogen, there was an

increased level of expression in spatially separated cells that are presumably proheterocysts. An increased level of *gfp* expression in proheterocysts was observed 12 hours after nitrate step down. Also, an increased level of *gfp* expression was seen in mature heterocysts. The interesting note here is the increased level of *gfp* expression in cell adjacent to the heterocysts. There was no difference in the level of expression or timing or the location in UHM129 in comparison to that in the wild type (Figure 16).

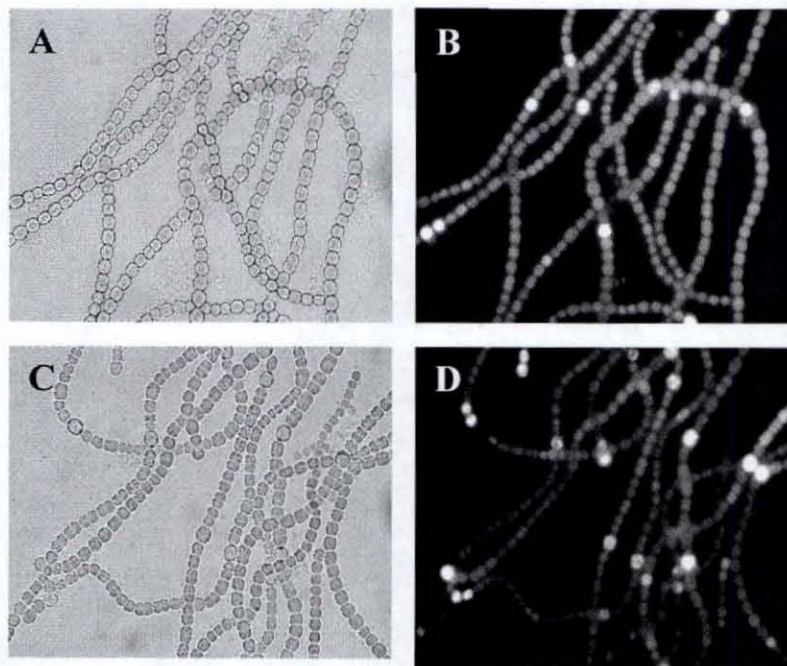


Figure 16. Expression of *hfq*. Promoter of *hfq* is expressed in proheterocysts, mature heterocysts and cells adjacent to mature heterocysts. Strain PCC 7120 carrying  $P_{hfq}$ -*gfp* 16h after induction of heterocysts formation under visible light (A) and ultra violet light (B). Strain PCC 7120 carrying  $P_{hfq}$ -*gfp* 40 h after induction of heterocysts formation under visible light (C) and ultra violet light (D)

## DISCUSSION

There has been extensive study conducted on Hfq in different organisms ranging from bacteria to humans. In most of the organisms studied Hfq is involved in various levels of regulation. In *E.coli*, even though an *hfq* knock out is not lethal, it seems to be affecting the normal functioning of the bacteria, including the growth rate (43). Evidence suggests that Hfq has a significant role in nitrogen fixation in nitrogen fixing bacteria such as *Azorhizobium caulinodans* and *Rhodobacter capsulatus*. The Hfq mutant in *Azorhizobium caulinodans* has a pleiotropic phenotype (23). The mutant produced nodules, however, it was unable to fix atmospheric nitrogen due to the inactivation of the *nif* genes. In *Rhodobacter capsulatus*, Hfq is not essential for growth under nitrogen replete conditions. But is required for maximal synthesis of nitrogenase and thus for optimal diazotrophic growth (11).

There is no apparent difference in the growth rate, nitrogen fixation or spacing and appearance of heterocysts in UHM 129. These results suggest the following scenarios: (a) Hfq might not have a role in pattern formation/decision making of heterocysts. (b) Another protein that can function in a similar way as Hfq, although no such protein was found in *Anabaena* to date, is carrying out the function of Hfq in UHM129.

However, increased expression of the promoter in proheterocysts and heterocysts suggests that it might have a role in heterocysts differentiation or function. Perhaps Hfq and similar proteins are regulating the expression of genes upregulated during heterocysts differentiation and this regulation fine tunes the

production of Het genes. However, it is not required for heterocyst function or differentiation. The phenotype of the mutant may be too subtle to be detected.

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