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# Transcriptional Regulation of Nitrogenase and Identification of a Fructose Transport System in Anabaena variabilis

Justin Lynn Ungerer
*University of Missouri-St. Louis*

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Justin Ungerer

**Advisory Committee**

Teresa Thiel
Wendy Olivas
Lisa Schechter
Bethany Zolman

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# ABSTRACT

Little is known about the regulation of nitrogenase genes in cyanobacteria. Transcription of the *nifH1* and *vnfH* genes, encoding dinitrogenase reductases for the heterocyst-specific Mo-nitrogenase and the alternative V-nitrogenase, respectively, was studied using a *lacZ* reporter. Two promoters were found to be responsible for expression of *nifHDK*; a weak promoter within *nifU1* and a strong promoter upstream of *nifB* (the *nifB1* promoter). The region immediately upstream of *vnfH* did not drive expression of *lacZ*, however the region that included the promoter for the upstream gene, *ava4055* did. Characterization of the previously reported *nifH1* and *vnfH* transcriptional start sites by 5' RACE revealed that these 5' ends resulted from processing of larger transcripts rather than by de-novo transcription initiation.

*Anabaena variabilis* grows heterotrophically using fructose while the close relative *Anabaena* sp. strain PCC 7120 does not. Introduction of a cluster of genes encoding a putative ABC-transporter, herein named *frtRABC*, into *Anabaena* sp. strain PCC 7120 on a replicating plasmid allowed that strain to grow in the dark using fructose, indicating that these genes were necessary and sufficient for heterotrophic growth. FrtR, a putative LacI-like regulatory protein, was essential for heterotrophic growth of both cyanobacterial strains. Transcriptional analysis revealed that the transport system was induced by fructose and that in the absence of FrtR, *frtA* was very highly expressed with or without fructose. In the *frtR* mutant, high-level expression of the fructose transporter resulted in cells that were extremely sensitive to fructose. *A. variabilis* grew better with increasing concentrations of fructose up to 50 mM, showing increased cell size and

heterocyst frequency. *Anabaena* sp. strain PCC 7120 did not show any of these changes when growing with fructose. Thus, although *Anabaena* sp. strain PCC 7120 could take up fructose and use it in the dark, fructose did not improve growth in the light.

# SECTION 1 : INTRODUCTION

## Cyanobacteria

Cyanobacteria occupy an important place in earth's history, constituting what is arguably the most important group of organisms to have inhabited the planet. As the only organisms to have evolved photosynthesis, a process that employs coupled photosystems that split water to harvest electrons while evolving molecular oxygen' cyanobacteria made possible the initial oxygenation of the earth's atmosphere (Flores, 2008). This event generated the earth's oxygen supply, which transformed the planet's redox environment in such a way that would lay the grounds for the existence of aerobic life as we know it (Kkarecha, 2005). To date, cyanobacteria remain the primary producers in marine environments, which constitute 70% of the earth's surface. Additionally, cyanobacteria were the photosynthetic associate in an early symbiotic partnership that introduced photoautotrophy into the eukaryotic kingdom; an event that contributed significantly to the existence of life on land (Kkarecha, 2005).

As primary producers cyanobacteria play a significant role in the carbon-cycle, especially in marine environments; and as nitrogen fixers they play a prominent role in the earth's nitrogen cycle. In fact, cyanobacteria produce nearly all of the biologically available nitrogen in marine environments (Davis, 2006; Montoya, 2004). The process of nitrogen fixation does not exist in any of the eukaryotic kingdoms; thus, even on land cyanobacteria contribute significantly to the biologically available nitrogen.

Compared to other bacteria, cyanobacteria display an unusually large diversity in morphology. Traditionally, taxonomic classification of cyanobacteria divides them into five groups based on development, physiology and morphology. It should be noted that

modern molecular phylogeny does not support many of these groupings (Flores, 2008). Group I (Chroococcales) is made up of the simplest unicellular cyanos that usually aggregate into colonies. This group reproduces by budding or binary fission and is incapable of cell differentiation (Rippka *et al.*, 1979). Group II (Pleurocapsales) are unicellular, colony-forming cyanobacteria that are characterized by a unique form of reproduction, multiple fission. This process involves multiple binary fissions within the cell wall, and without growth of the cell, to generate from 4-1000 spore like cells called baeocytes; which are subsequently released upon rupture of the cell wall (Rippka *et al.*, 1979). The spores then regenerate cell walls and form mature cells, which remain tightly adhered to each other. Group III (Oscillatoriales) are true multicellular filamentous cyanobacteria. This group reproduces by binary fission in a single plane that generates filaments one cell thick. Cyanobacteria from this group are incapable of differentiating alternative cell types (Komarek J., 2005). Group IV (Nostocales) are multicellular, filamentous cyanobacteria that are capable of differentiating multiple cell types including heterocysts, akinites, and hormogonia (Rippka *et al.*, 1979). Members of this group also reproduce by binary fission occurring in a single plane, which generates linear filaments one cell thick. Group V (Stigonematales) are multicullular, cell differentiating cyanobacteria that utilize more complex cell arrangements such as branching filaments. Cell division occurs in multiple planes yielding branched filaments in these strains (Rippka *et al.*, 1979).

### *Anabaena*

The studies contained here focus primarily on the cyanobacterium *Anabaena variabilis* ATCC 29413 strain FD referred to hereafter as *A. variabilis*. Strain FD is an

isolate of *A. variabilis* that grows well at 40° C, is more resistant to phage growth, and more amendable to genetic manipulations (Currier and Wolk, 1979). *Anabaena* sp. 7120 is often compared to *A. variabilis* in this discussion. *Anabaena* sp. 7120 is a close relative to *A. variabilis*, as these two strains share 95% amino acid identity between their coding regions. Both *A. variabilis* and *Anabaena* sp. 7120 have complete genome sequences that further facilitate genetic studies of these organisms. The genome of *A. variabilis* is 6.3 Mbp, coding for approximately ~5900 proteins; while the genome of *Anabaena* sp. 7120 is 5.4 Mbp, containing ~5300 genes (NCBI). Both *Anabaena* strains are Group IV cyanobacteria from the class Nostocales. *Anabaena* grows in linear filaments and is capable of differentiating various specialized cell types depending on environmental conditions. In the absence of a fixed nitrogen source *Anabaena* will differentiate specialized cells called heterocysts that fix atmospheric dinitrogen. When exposed to unfavorable environmental conditions such as desiccation, cold, or phosphate-deprivation, *Anabaena* will develop spore-like cells called akinetes, which serve as survival structures (Elfgren, 2003). *A. variabilis* is also capable of differentiating a third cell type called hormogonia. These cells utilize gliding motility to establish infection of its symbiotic partners.

Because of its complex life cycle, *Anabaena* is often studied as a model organism for development and cellular differentiation. Also, *Anabaena* is actively studied for its potential as a renewable energy source because it can split water and evolve hydrogen using only sunlight as the energy source. Finally, *Anabaena* is studied as a model for photosynthesis because it uses both plant like photosystems, however *Anabaena* is much easier to genetically engineer and study than plant chloroplasts. Furthermore, *Anabaena*

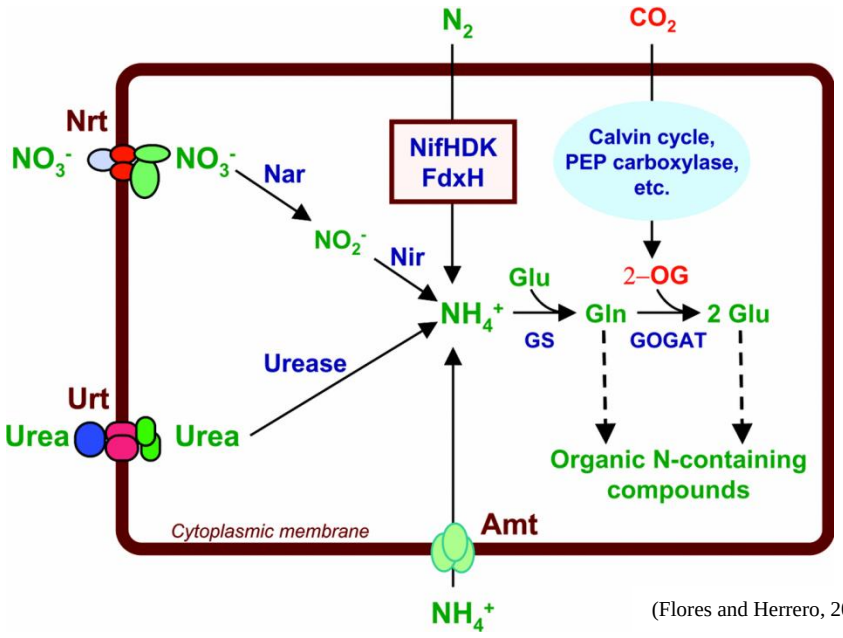
*variabilis* can grow heterotrophically in the dark with fructose, thus it is possible to make novel photosystem mutations that would be lethal in higher organisms (Nyhus *et al.*, 1993).

## Nitrogen assimilation

Cyanobacteria are capable of utilizing a variety of nitrogen sources including inorganic ions such as ammonium, amino acids such as glutamine, or molecular nitrogen via nitrogen fixation (Herrero *et al.*, 2001). Nitrogen assimilation in cyanobacteria can be compared to carbon assimilation in heterotrophic bacteria in the following way: Heterotrophic bacteria possess metabolic pathways for the utilization of a wide variety of carbon sources; however the choice of a carbon source is highly regulated. Generally, all pathways for carbon assimilation are repressed except for that of the preferred carbon source. Similarly, cyanobacteria are capable of utilizing a wide variety of nitrogen sources, and if various nitrogen sources are present, they will repress genes except those for utilization of the preferred source (Herrero *et al.*, 2001).

Ammonium is the preferred nitrogen source for cyanobacteria (Flores and Herrero, 2005); consequently utilization of alternative nitrogen sources is repressed in the presence of ammonium. These nitrogen sources include nitrate, nitrite, urea, glutamine, cyanate and dinitrogen. Dinitrogen is the least preferred nitrogen source and is only assimilated in the absence of all other combined nitrogen sources. Ammonium can readily cross the cyanobacterial membranes at the basic pH that most cyanobacteria thrive (Boussiba and Gibson, 1991); however all cyanobacteria sequenced thus far have ammonium transport genes (*amt*) (Figure 1.1) (Boussiba and Gibson, 1991; Kashyap and

Johar, 1984; Markowitz *et al.*, 2006). This ensures that ammonium is efficiently scavenged from the environment, even when it is present in low concentrations.




(Flores and Herrero, 2005)

**Figure 1.1** Nitrogen assimilation pathways in *Anabaena*. $N_2$ $NH_4^+$, $NO_3^-$ and urea are reduced to ammonium which is then assimilated through the GS-GOGAT pathway.

The assimilation of nitrate and nitrite occurs through a single pathway mediated by a pair of ferredoxin containing reductases (Frías *et al.*, 1997; Kumar and Singh, 1985), *narB* and *nirA* (Figure 1.1), and only in the absence of ammonium. The two nitroreductases work sequentially to reduce nitrate to nitrite then to ammonium. In *Anabaena*, nitrate-nitrite uptake is handled by a specific ABC-type transporter, which is the product of the *nrtABCD* genes (Frías *et al.*, 1997). Similarly, urea is taken up by its own ABC-type transporter and is metabolized by the urease genes (Figure 1.1), *ureABC*, which are homologous to other typical urease systems found in *E. coli* (Flores and Herrero, 2005).

Ultimately, all nitrogen sources are converted to ammonium before they are assimilated (Flores and Herrero, 2005). Assimilation of ammonium occurs through the

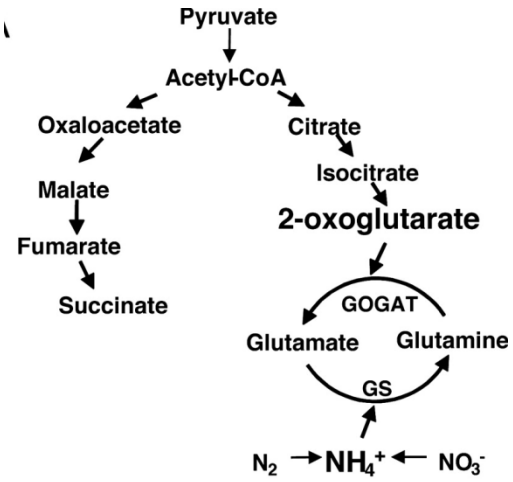



**Figure 1.2** The cyanobacterial Krebb cycle and its interaction with the GS-GOGAT cycle. Cyanobacteria lack a complete Krebb cycle, thus 2-OG is used as a carbon skeleton for nitrogen assimilation

(Laurent *et al.*, 2005)

glutamine synthetase-glutamate synthase pathway (GS-GOGAT) (Meeks and Galonsky, 1977; Stacey *et al.*, 1979). *Anabaena* employs multiple N-regulated glutamine synthetases such as GlnA (Merida *et al.*, 1992) and GlnB (Forchhammer and Tandeau de Marsac, 1995). In contrast, *Anabaena* utilizes only a single glutamate synthase, GlsF (Martin-Figueroa *et al.*, 2000; Zhou *et al.*, 2008), which is not N regulated. The carbon skeleton through which ammonium is assimilated is 2-oxoglutarate. Cyanobacteria lack a key enzyme in the Krebs cycle, 2-oxoglutarate dehydrogenase (Martin-Figueroa *et al.*, 2000), thus 2-oxoglutarate is used primarily for biosynthesis of glutamate-containing compounds resulting from the glutamine synthetase-glutamate synthase pathway (Figure 1.2). The ratio of 2-oxoglutarate to glutamate varies as a function of the C:N balance of the cell; thus, 2-oxoglutarate levels serve as a sensor of the nitrogen status of the cell.

## Nitrogen Fixation

Nitrogen is required for the biosynthesis of most organic compounds that comprise living things including nucleic acids, proteins, cell walls, and chlorophyll.

Although $N_2$ is the largest component of the earth's atmosphere, biologically available nitrogen is commonly a limiting factor for growth in most ecosystems (Triplett, 2000). Most of the earth's $N_2$ supply is biologically unavailable because the triple bond of dinitrogen is very stable thus free nitrogen is not readily available (Igarashi and Seefeldt, 2003). There are only a few organisms, all of which are prokaryotic, that have the ability to reduce dinitrogen to ammonium. This process is known as nitrogen fixation; and the organisms that perform this process are known as diazotrophs.

Approximately two-thirds of the nitrogen fixed globally is performed biologically (Rubio and Ludden, 2005). Furthermore, half of the biological nitrogen fixation worldwide occurs in the ocean with cyanobacteria being exclusively responsible for this activity (Fay, 1992). On land, nitrogen fixation is carried out by a variety of prokaryotes that occupy diverse niches, and in many cases, form symbiotic relationships with plants or animals (Adams and Duggan, 2008; Burris and Roberts, 1993). Nitrogenase, the only enzyme responsible for nitrogen fixation, is present in relatively similar forms across all genera (Burris and Roberts, 1993).

## Structure of Nitrogenase

Nitrogenase is a highly-conserved enzyme complex in diverse prokaryotes including both *Bacteria* and *Archaea* (Zehr *et al.*, 1998). The close physical and chemical characteristics shared by all nitrogenases, even in dissimilar organisms, suggest that this enzyme evolved once in an ancient ancestor common to *Archaea* and *Bacteria*, and has remained relatively unchanged (Emerich and Burris, 1978).

The nitrogenase complex is composed of two components, the Fe component known as the dinitrogenase reductase (*nifH*) and the MoFe component known as dinitrogenase (*nifDK*) (Figure 1.3). The dinitrogenase is a $\alpha_2\beta_2$ tetramer composed of two *nifDK* heterodimers and each dimer contains two cofactors, the P cluster [8Fe-7S] and MoCo-cofactor [7Fe-Mo-9S-homocitrate] (Triplett, 2000). The dinitrogenase reductase functions as a homodimer with a [4Fe-4S] cluster housed at the interface between the subunits (Fay, 1992). Each dinitrogenase reductase component has two MgATP binding sites, one on each subunit. The nitrogenase is composed of 2 dinitrogenase reductase homodimers bound to a dinitrogenase heterotetramer (Figure 1.3).

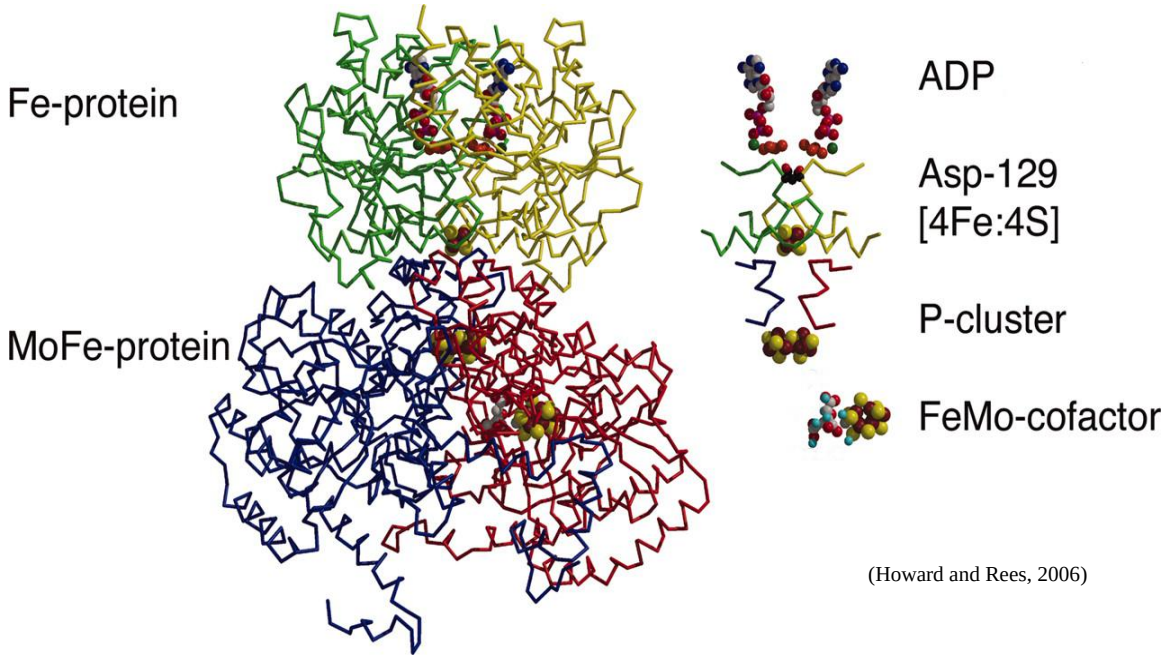



**Figure 1.3** Structure of the nitrogenase complex. Shown on the left is a $NifH_2DK$ tetramer with cofactors (this structure represents only half of the nitrogenase). Shown on the right is the arrangement of the cofactors within the protein. NifH dimer is shown in yellow and green. The NifDK dimer is shown in blue and red.

## Mechanism of the nitrogenase

The mechanism of nitrogenase action is as follows (reviewed in Burgess and Lowe, 1996; Leigh, 2004) (Figure 1.4). The cycle begins with the Fe protein (NifH) bound to 2 MgATP and its [4Fe-4S] clusters in a reduced state. A NifH dimer binds to each half of the NifDK tetramer resulting in the transfer of a single electron from the [4Fe-4S] cluster of $NifH_2$ to the P cluster of NifDK (Leigh, 1995). The electrons are then funneled from the P cluster of NifDK to the MoFe cofactor of NifDK, changing its oxidation state by -1 (Figure 1.4) (Leigh, 1995). Hydrolysis of two MgATP is required to release $NifH_2$ from NifDK, at which time it exchanges MgADP for fresh MgATP, and its [4Fe-4S] cluster is rereduced by a ferredoxin or flavodoxin (Igarashi and Seefeldt, 2003).

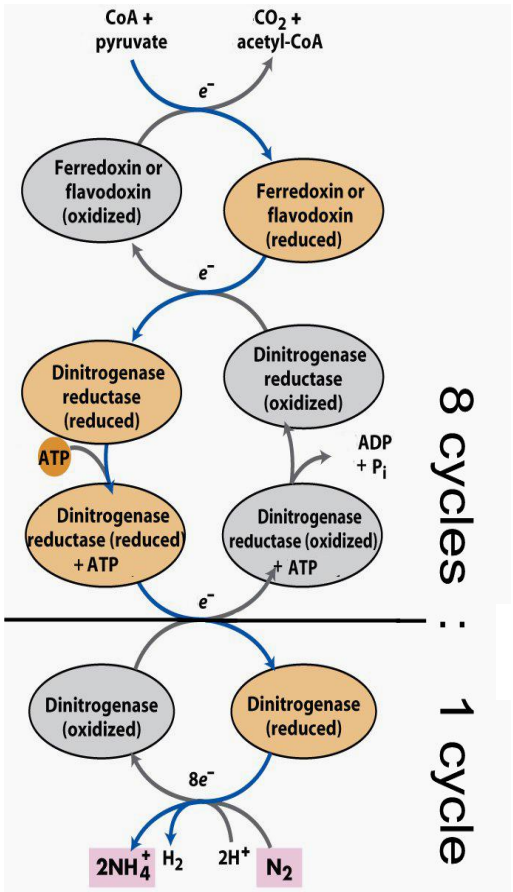



**Figure 1.4** Schematic of cyclic reduction of the dinitrogenase by the dinitrogenase reductase. The dinitrogenase sequentially transfers 8 electrons to the dinitrogenase. Each transfer requires hydrolysis of ATP and disassociation of NifH from NifDK, followed by rereduction of the dinitrogenase reductase. The blue arrows represent the flow of electrons.

Modified from
http://www.blc.arizona.edu/courses/bioc462b/grimes/nitrogen06/introduction.cfm

The MoFe-cofactor is the site where the reduction of dinitrogen to ammonium occurs; however the MoFe-cofactor must accumulate eight electrons before it can reduce a single dinitrogen (Figure 1.4) (Leigh, 1995). Each electron is transferred to the MoFe -cofactor sequentially by NifH as described above, and each electron transferred requires the hydrolysis of 2 ATP. Through this process the MoFe-cofactor functions as a sink to accumulate reducing equivalents until it has stored enough potential energy to break the triple bond of dinitrogen (Figure 1.4). The overall reduction of $N_2$ to $NH_4^+$ has a negative enthalpy ($\Delta H^0$ = -45.2 kJ $mol^{-1}$), however due to the stability of the $N_2$ triple bond this reaction has an overwhelming energy of activation ($E_{a=}$ 420 kJ $mol^{-1}$) (Modak, 2002). For this reason, nitrogenase carries out one of the most energetically expensive metabolic reactions known (Grula, 2005), with the reduction of each dinitrogen molecule requiring 8 electrons and 16 ATP.

## Alternative nitrogenages

The Mo-dependent nitrogenase is the principal nitrogenase for all nitrogen-fixing bacteria. A few bacteria also employ alternative nitrogenases that are very similar to Mo nitrogenases except that they utilize variations of the MoFe-cofactor. The cofactors differ only in that a vanadium or iron atom replaces the Mo atom of the MoFe-cofactor. *A. variabilis* has two nitrogenases in addition to the primary Mo nitrogenase (Thiel, 1993; Thiel *et al.*, 1995).

The Nif2 nitrogenase in *A. variabilis* is homologous to the *nif1* nitrogenase and also utilizes a MoFe cofactor (Thiel *et al.*, 1995; Thiel *et al.*, 1997). The Nif2 nitrogenase is repressed under aerobic growth conditions and in the presence of a fixed

nitrogen source; however, under anaerobic growth conditions this system is expressed in all vegetative cells (Thiel *et al.*, 1995; Thiel *et al.*, 1997). This contrasts to the *nif1* genes, which are expressed only in heterocysts under aerobic growth conditions. The third nitrogenase of *A. variabilis* is the Vnf nitrogenase, which uses a vanadium atom in place of the molybdenum atom in the cofactor (Thiel, 1993). This nitrogenase is expressed only under conditions of nitrogen deficiency combined with severe Mo limitation, thus it is Mo and N repressed (Thiel, 1993). Due to a high affinity Mo uptake system in *A. variabilis*, expression of the V-nitrogenase does not occur unless the Mo concentration is $10^{-9}$ M or less (Thiel *et al.*, 2002; Zahalak *et al.*, 2004). It is likely that during the course of evolution tight control of the *vnf* genes has been beneficial because it is less efficient than the Mo-nitrogenase.

All known nitrogenases are obligate hydrogenases, reducing protons to form hydrogen (Smith *et al.*, 2003). The efficient Mo-nitrogenase allocates ~75% of the electron flux to the reduction of nitrogen, losing only 25% of its electrons to the reduction of protons. In contrast, the V-nitrogenase loses half of its electrons to proton reduction while the Fe-nitrogenase allocates ~80% of its reducing equivalents to the reduction of protons' reducing very little nitrogen (Masepohl, 2002; Zhao *et al.*, 2006). The higher rates of $H_2$ evolution by the alternative nitrogenases suggests that protons are

**Figure 1.5**

Mo Nitrogenase - $N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$

V Nitrogenase - $N_2 + 12\ H^+ + 12\ e^- + 40\ ATP \rightarrow 2\ NH_3 + 3H_2 + 40\ ADP + 40\ P_i$

Fe Nitrogenase - $N_2 + 24\ H^+ + 24\ e^- + ?\ ATP \rightarrow 2\ NH_3 + 9H_2 + ?\ ADP + ?\ P_i$

Modified from (Masepohl, 2002)

preferentially reduced over dinitrogen resulting in energy lost in the form of dihydrogen (Leigh, 2004). Figure 1.5 is a comparison of the reactions catalyzed by the three nitrogenase systems (Bolin, 1993 ; Masepohl, 2002). Notice that in all cases only a single dinitrogen is reduced; however, the alternative nitrogenases require more energy input in the form of MgATP and reducing equivalents than does the Mo-nitrogenase. Because these enzymes typically have higher ratios of $H_2$ to $NH_4$ production than Mo-dependent nitrogenases, they have an increased potential for hydrogen production.

## Co-factor

The MoFe-cofactor is located in the nitrogenase active site, which is the site of reduction of dinitrogen to ammonium (Igarashi and Seefeldt, 2003b). This cofactor is described as a [Mo-$Fe_2$-$S_3$X] cubane, and a [$XFe_4S_3$] cubane sharing a common X atom

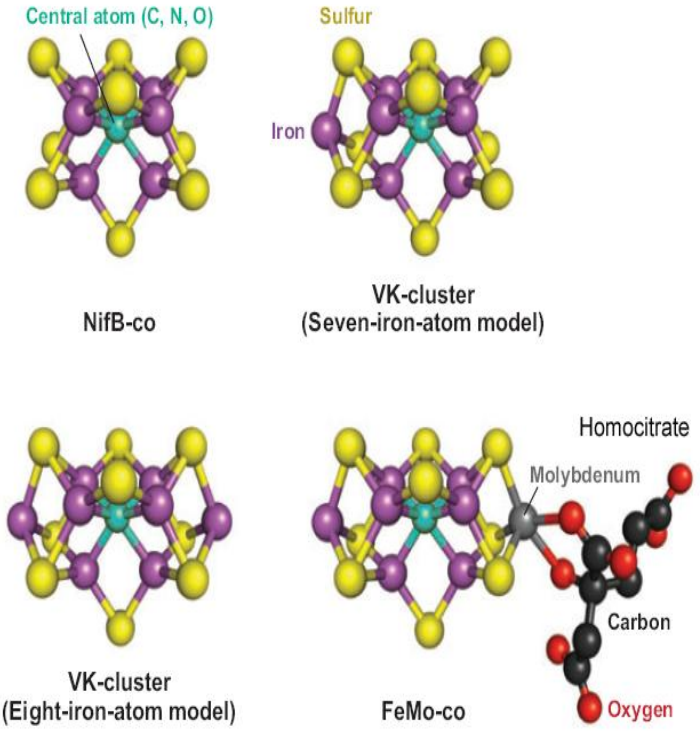



**Figure 1.6** Structure of the NifB-Co, VK cluster, and MoFe cofactor.

(Rubio and Ludden, 2008)

at one corner (Figure 1.6) (Chan *et al.*, 1993; Einsle *et al.*, 2002). The central atom has yet to be elucidated; however studies indicate that it is an O, N, or C atom. A homocitrate group is also bound the Mo atom of the cofactor (Figure 1.6).

Iron-sulfur clusters are common in enzymes catalyzing a variety of biochemical reactions including photosynthesis, respiration, nitrogen fixation, and numerous other redox reactions (Johnson *et al.*, 2005). The MoFe-cofactor is considered the most complex iron-sulfur cluster that is synthesized biologically (reviewed in Rubio and Ludden, 2008). Consequently, no less than 12 Nif proteins are involved in the synthesis of this cofactor; most of which are rapidly inactivated by oxygen, as are the various cofactor intermediates (Rubio and Ludden, 2005). Most of the proteins and MoFe-cofactor intermediates have been successfully purified and used for synthesis of the mature cofactor in vitro, which has greatly increased our understanding of the biosynthetic pathway that produces the MoFe-cofactor (Rubio and Ludden, 2008). These proteins can be subdivided into groups with three general functions: Molecular

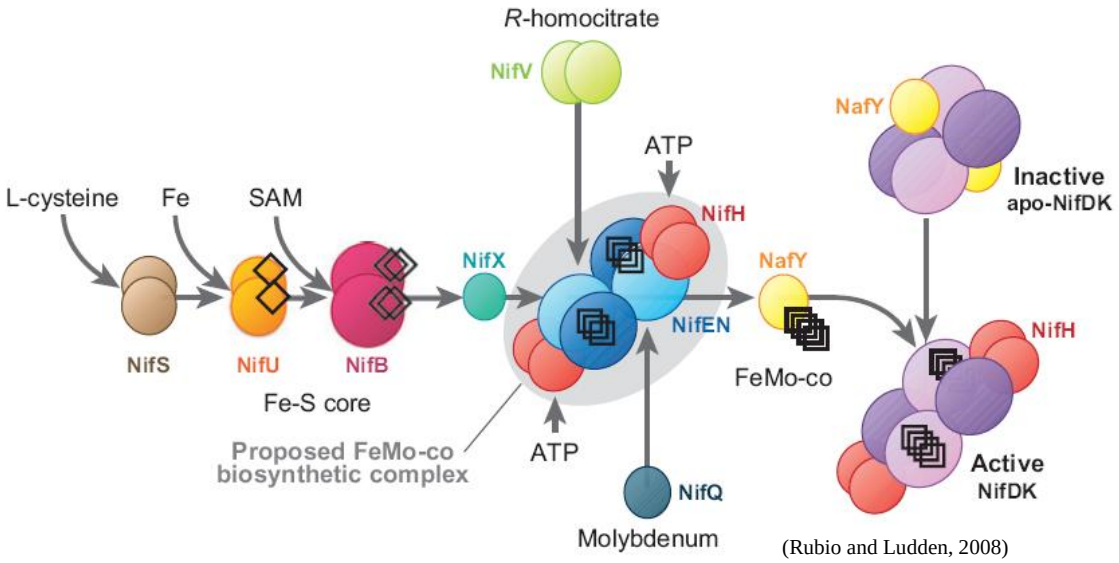



**Figure 1.7** Current model of biogenesis of the FeMo-cofactor and holonitrogenase complex.

scaffolding on which the cofactor is assembled (NifB, NifU, NifEN), carrier proteins that shuttle the cofactor between scaffolding proteins (NifX, NafY), and enzymes that provide metal atoms to the scaffold for the construction of the cofactor (NifS, NifQ, and NifV).

The NifS and NifU proteins play a role in biosynthesis of the FeMo-cofactor and the P clusters of the dinitrogenase, as well as the [$Fe_4$-$S_4$] cluster of the dinitrogenase reductase (Jacobson *et al.*, 1989; Johnson *et al.*, 2005). NifS is a cystine desulfurase (Zheng *et al.*, 1993) that catalyzes the desulfuration of L-cystine, yielding L-alanine (Zheng *et al.*, 2002) and a sulfur atom that is transferred onto the initial scaffold, NifU (Figure 1.7) (Dos Santos *et al.*, 2007; Yuvaniyama *et al.*, 2000; Zheng *et al.*, 1998). NifU forms a transient heterodimer with NifS, accepting the sulfur atom from NifS and forming simple [Fe-S] clusters (Yuvaniyama *et al.*, 2000). NifU can coordinate 4 [Fe-S] clusters simultaneously (Agar *et al.*, 2000) and catalyzes the condensation of these into a [$Fe_4S_4$] cluster (Fu W, 1994). The [$Fe_4S_4$] cluster is then transferred from NifU onto the next molecular scaffold in the pathway, NifB (Figure 1.7) (Curatti *et al.*, 2007). NifB catalyses the transformation of the [$Fe_4S_4$] cluster from NifU into NifB-cofactor (Shah VK, 1994), which is the next intermediate in the biosynthesis of MoFe-cofactor. The specific structure of NifB-cofactor is unknown however electron paramagnetic resonance spectroscopy (EPR) suggests that it is a [$Fe_6S_9$] cluster (Figure 1.6) (Yisong *et al.*, 2006).

NifX removes the NifB-cofactor from the NifB protein and deposits it onto the final molecular scaffold, NifEN (Figure 1.7) (Hernandez *et al.*, 2007). NifEN is a $\alpha_2\beta_2$ heterotetramer (Suh *et al.*, 2002) whose proteins share a high degree of sequence similarity to NifDK proteins of the dinitrogenase (Hu *et al.*, 2009). It is believed that the NifEN protein complex forms a scaffold resembling the NifDK dinitrogenase on which

the mature FeMo-cofactor is assembled (Brigle *et al.*, 1987). Additionally, assembly of the mature FeMo-cofactor requires NifH binding to NifEN with transfer of electrons to NifEN in a similar manner to the interaction of NifH with NifDK (Brigle *et al.*, 1987; Hu *et al.*, 2006a). NifEN catalyzes the transformation of NifB-cofactor into the mature FeMo-cofactor by facilitating three reactions (Hu *et al.*, 2006a; Rubio and Ludden, 2008).

First, NifEN catalyzes the incorporation of an additional iron atom into NifB-cofactor, yielding the VK cluster (Figure 1.6) (George *et al.*, 2007), a [$Fe_{7\text{ or }8}S_9$] cluster. The second reaction that is catalyzed by NifB is the addition of a Mo atom to the VK cluster (Figure 1.6) (Corbett *et al.*, 2006; Rubio and Ludden, 2008) (Figure 1.6). This reaction occurs in two steps. First, NifQ donates the Mo atom to NifEN (Figure 1.7) (Hernandez *et al.*, 2008; Imperial *et al.*, 1984). Subsequently NifH must bind NifEN with hydrolysis of MgATP to supply reducing energy for the incorporation of the Mo atom into the VK cluster, forming a [$MoFe_6S_9$] cluster (Hu *et al.*, 2005; Hu *et al.*, 2006b). The third reaction that is carried out on the NifEN scaffold is the addition of the homocitrate group to form the mature FeMo-cofactor (Figure 1.7) (Rangaraj and Ludden, 2002), a reaction that also requires the activity of NifH (Rubio and Ludden, 2008). The homocitrate is delivered to NifEN by the protein NifV, however details of this reaction remain unclear. NifV is a homocitrate synthetase that catalyzes the condensation of acetyl-coA and $\alpha$-ketoglutarate to form homocitrate (Zheng *et al.*, 1997), however NifV is also required for incorporation of mature MoFe-cofactor into the apo-nitrogenase in vitro (Rubio and Ludden, 2008).

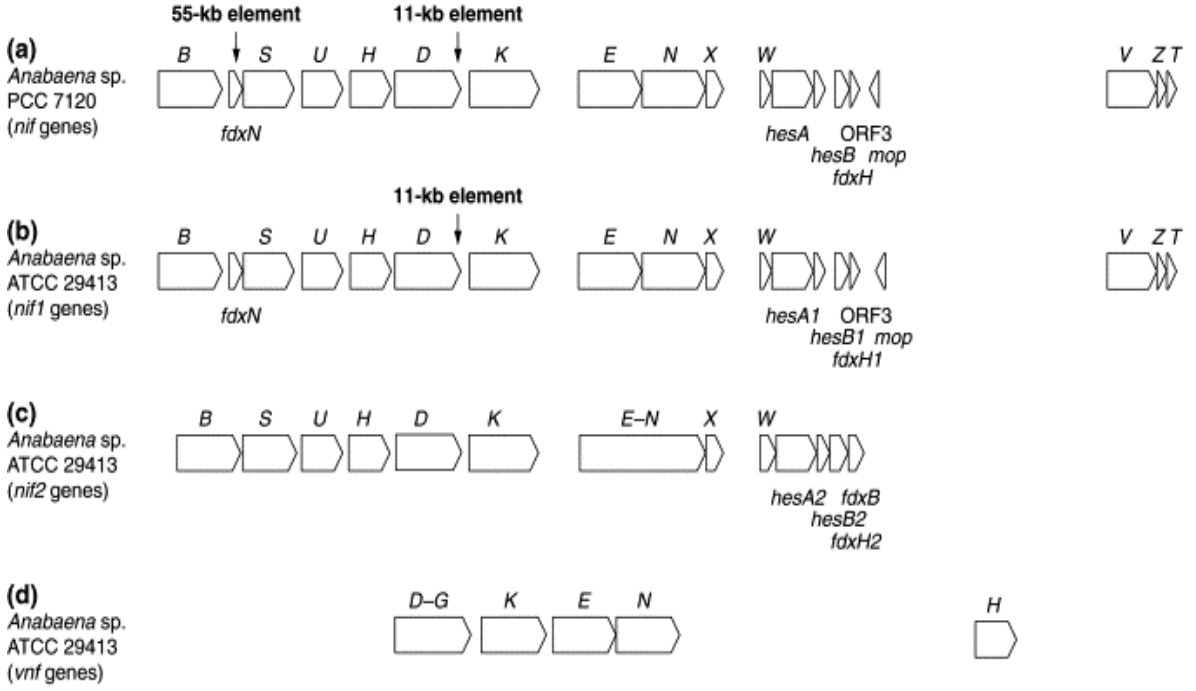
NafY ($\gamma$) is the final carrier protein that is involved the biosynthesis of the FeMo-cofactor. NafY functions to carry the mature FeMo-cofactor from NifEN to NifDK and

to facilitate the insertion of FeMo-cofactor into NifDK (Figure 1.7) (Homer *et al.*, 1995; Rubio *et al.*, 2004). NafY has two domains whose functions have been separated by expression and purification of each domain separately. One domain of NafY binds FeMo-cofactor tightly while the other domain binds NifDK tightly (Rubio *et al.*, 2002). It is hypothesized that NafY carries the cofactor to NifDK and stabilizes a conformation of apo-nifDK that is amendable to the insertion of the FeMo-cofactor (Rubio *et al.*, 2002).

## Organization of the *nif* genes

Of particular interest is the high degree of similarity between the arrangements of *nif* gene clusters that has been retained by many heterocyst-forming, nitrogen-fixing cyanobacteria, which suggests that there may be a function for these arrangements (Böhme, 1998). The genes involved in nitrogen fixation are typically clustered tightly in a chromosomal region that is often referred to as a "*nif* island". All genes on the *nif* island are nitrogen regulated, and depending on the *nif* island, either Mo regulated or $O_2$ regulated too. In *Anabaena* spp. the *nif1* island spans about 24 kb and includes ~16 genes within 5 operons (Markowitz *et al.*, 2006). The most prominent transcripts from this region are *nifB-fdxN-nifSU, nifHDK, nifENX, nifW-hesAB-fdxB,* and *nifVZT* (Figure 1.8). There are also several small (<50aa) orfs within this region that appear to be N- regulated and are conserved, but for which no function has been ascribed.

The *vnf* island of *A. variabilis* does not share the same arrangement as the genes for the Mo-nitrogenases (Thiel, 1993, 1996). Additionally, many genes involved in the biosynthesis of the cofactor are missing from the *vnf* island (Thiel, 1993, 1996). The VFe cofactor is made by the same Nif1 proteins that are responsible for the biogenesis of the MoFe-cofactor; thus, it is not necessary for the *vnf* island to contain additional copies of these genes (Thiel, 1993). It is not understood how the same cofactor biogenesis machinery is capable of generating both the MoFe-cofactor and the VFe-




(Böhme, 1998)

**Figure 1.8** Arrangement of various *nif* islands in heterocyst forming cyanobacteria.

cofactor, depending on which nitrogenase the cells are using. Another, big difference between the *nif* and *vnf* gene clusters is the location of *nifH*. *nifH* is usually found on the same operon as the other two structural components, *nifDK*. In the *vnf* island, *vnfH* is separated from the other *vnf* genes and phylogenetic analysis indicates that *vnfH* may have come from a different organism than the other *vnf* genes (IMG). Additionally, NifH can substitute for VnfH in a *nifH* mutant (Pratte *et al.*, 2006), suggesting that the *vnfH* is not essential to the function of the V-nitrogenase.

## DNA rearrangements

Another characteristic of *nif* islands in cyanobacteria, especially heterocyst-forming cyanobacteria, is the presence of large DNA elements that interrupt the operons of a several key genes. In *A. variabilis* there is an 11-kb DNA element interrupting the ORF of the nitrogenase structural gene, *nifD* (Brusca *et al.*, 1989; Henson *et al.*, 2008). A closely related species, *Nostoc punctiforme*, has a 24-kb DNA element in *nifD* (Henson *et al.*, 2005). Interestingly, the two excision elements are in the exact same location in *nifD*, however there is no discernible similarity between the two DNA elements. In several species there is also a 55-kb excision element interrupting the *nifBSU* operon (Golden *et al.*, 1988; Golden and Ramaswamy, 1992); however, this element is absent in *A. variabilis*. A 9.5-kb excision element that is present in the heterocyst-specific hydrogenase gene *hupL* of *Anabaena.* sp. 7120 is also absent in *A. variabilis* (Carrasco *et al.*, 1995; Carrasco *et al.*, 1998).

The excision elements are removed during heterocyst development, restoring the coding regions of their respective genes only in heterocysts. Removal of the excision

elements is a developmentally regulated process that occurs late in heterocyst differentiation (Carrasco *et al.*, 1994), just prior to nitrogenase expression. The excision elements are removed via site-specific recombination that is mediated by site-specific recombinases. The recombinases are encoded by a gene on the DNA element that they excise (Brusca *et al.*, 1989; Carrasco *et al.*, 1994; Carrasco and W., 1995; Ramaswamy *et al.*, 1997) and each DNA element contains a specific recombinase that removes only its coordinating DNA element (Haselkorn, 1992).

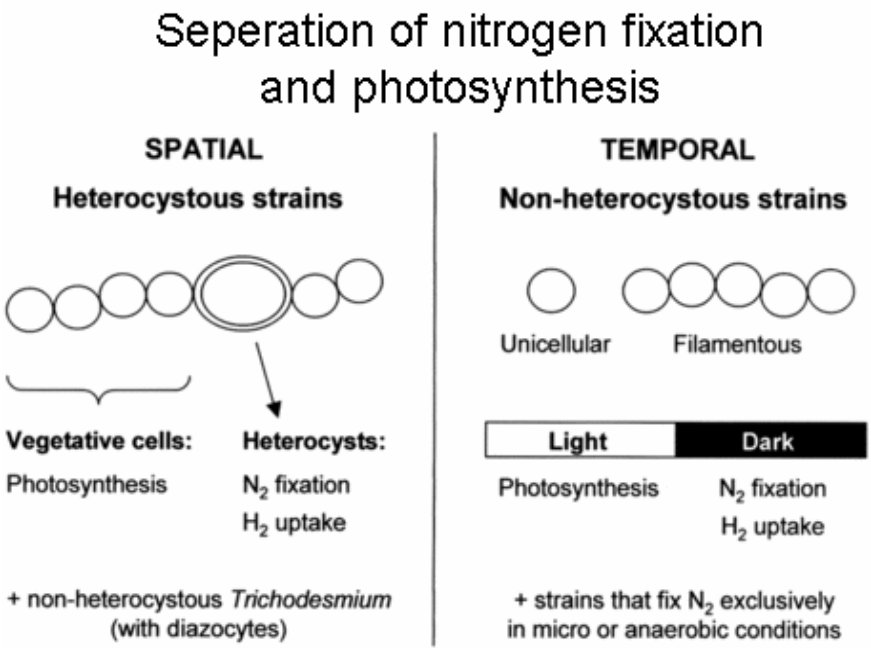
The exact function of the excision elements is unclear, however it is hypothesized that they serve to prevent aberrant transcription of the genes they interrupt in vegetative cells (Golden and Ramaswamy, 1992). It is believed that aberrant transcription will run into the large DNA element at which point it is terminated and/or degraded because it is not being translated. Strains lacking the excision element have been generated by expression of their respective excisases in *trans*, which causes loss of the excision element in vegetative cells (Brusca *et al.*, 1990). Characterization of these strains has provided little insight into the purpose of the excision elements because expression of the *nif* genes is undetectable in vegetative cells, even in strains lacking the excision elements. Furthermore, growth assays on strains lacking excision elements show no impairment in diazatrophic growth, vegetative growth ($+NH_4$), or heterocyst differentiation (Brusca *et al.*, 1990; Golden and Wiest, 1991). Thus, although the conservation of different elements in the exact same location in *nifD* suggests an important function for the element, the excision elements are dispensable.

## Protection of the nitrogenase

Nitrogenase enzyme evolved well before the oxygenation of the earth's atmosphere and oceans (Fay, 1992b). This resulted in an enzyme that was not designed to work in the presence of oxygen. Consequently, nitrogenase is readily inactivated by oxygen (Dingler and Oelze, 1985). Furthermore, the Fe-S clusters of nitrogenase and associated biosynthetic proteins are also irreversibly inactivated in the presence of oxygen, rendering them useless. Cyanobacteria, their photosystems, and their nitrogenases have co-evolved as the oxidation state of the planet transitioned from a reducing anaerobic to an oxidizing aerobic environment. Not only have cyanobacteria had to overcome the hurdle of fixing nitrogen in an aerobic environment, they have had to reconcile the coexistence of oxygen-sensitive nitrogenase with oxygen-evolving photosynthesis in the same cell.

There are two general strategies that cyanobacteria have adopted to allow them to overcome these problems. The solution is either temporal or spatial segregation of nitrogen fixation and photosynthesis (Figure 1.9). Many unicellular and nonheterocystous filamentous cyanobacteria coordinate these two processes based on the diurnal cycle, in many cases using circadian rhythms to regulate the process (Fay, 1976). Nitrogen fixation is carried out in the dark period when photosynthetic oxygen evolution is minimal.

Alternatively, heterocystous cyanobacteria have acquired the ability to partition nitrogen fixation and photosynthesis into separate cells (Fay and Walsby, 1966). This is the essential function of heterocysts. Heterocysts employ several mechanisms to protect the nitrogenase. First they have a thick envelope that limits oxygen diffusion into the cell. They inactivate the oxygen evolving photosystem II (Katoh and Ohki, 1975); and exhibit significantly increased respiration that serves to scavenge internal oxygen (Hochman and Burris, 1981; Poole and Hill, 1997) as well as provide energy for nitrogen fixation (Peterson, 1989). Heterocysts also up-regulate genes involved in protection from reactive oxygen species such as superoxide dismutase, peroxidase and catalase (Dingler and Oelze, 1987) as well as several antioxidant enzymes (Tel-or *et al.*, 1986).



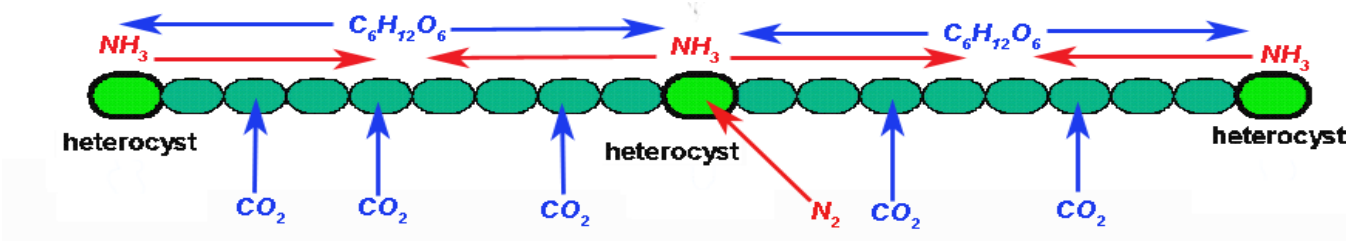

(Tamagnini *et al.*, 2005)

**Figure 1.9** Nitrogen fixation and photosynthesis cannot occur simultaneously. These two processes are separated spatially or temporally

## Heterocysts

Heterocysts are highly specialized cells that provide an anaerobic microenvironment for the specific purpose of nitrogen fixation. Heterocysts are terminally differentiated, non-dividing cells that are morphologically and physiologically distinct from vegetative cells. Heterocysts are semi-regularly spaced along a filament, appearing every 10-15 cells. Heterocysts function to provide fixed nitrogen for the vegetative cells in a filament, while receiving fixed carbon and reductant in return (Figure 1.10). Metabolites are transported between heterocysts and vegetative cells by route of their periplasmic space (Mariscal *et al.*, 2007). In a filament, each cell contains its own inner membrane and pepdigoglycan layer, however the outer membrane surrounds the entire filament rather than individual cells (Flores *et al.*, 2006; Mariscal *et al.*, 2007; Nicolaisen *et al.*, 2009b). Thus, the periplasmic space is continuous between cells and can serve as a conduit through which metabolites are exchanged (Flores *et al.*, 2006).

The most obvious characteristic of heterocysts is their larger size compared to vegetative cells due to the existence of distinct layers of protective envelope that are deposited external to the cell wall (Weckesser and Jürgens, 1988). The thick heterocyst envelope serves to limit oxygen diffusion into the cell. During the course of heterocyst development, two envelope layers are deposited outside the cell wall. The inner layer is the glycolipid layer, which limits the diffusion of oxygen into the cell; and the outer layer is a thick polysaccharide layer, which is believed to protect the fragile glycolipid layer (Figure 1.11) (Adams and Carr, 1989).




Modified from http://biology.ucsd.edu/faculty/jgolden.html

**Figure 1.10** Seperation of carbon fixation and nitrogen fixation within a filament of heterocyst forming cyanobacteria

## Glycolipid layer

Mutant strains that lack the glycolipid layer can fix nitrogen only under anaerobic conditions, indicating that the glycolipid layer is essential to limiting oxygen diffusion into the cell (Figure 1.11) (Lambein, 1973; Walsby, 1966). The glycolipid layer, which is just external to the cell wall, is composed primarily of hexose derivatives of long chain polyhydroxyketones (Bauersachs *et al.*, 2009). Two specific glycolipids have been identified from this layer. The major component is HGL1 (1-(O-α-D-glucopyranosyl)-3-keto-25-hexacosanediol) and the minor component is HGL2 (1-(O-α-D-glucopyranosyl)-3-keto-25-hexacosanol) (Gambacorta, 1996).

HglC, HglE, and DevBCA are the only proteins that have been implicated directly in the synthesis of the glycolipids (Fan *et al.*, 2005); however, these are not the only proteins involved in this process. Two ABC-type transporters are coded by *devABC* (Fiedler *et al.*, 1998; Fiedler *et al.*, 2001) and the *hglBC* gene products are responsible for export of the glycolipids and assembly factors into the periplasmic space. *hgdD* encodes Heterocyst Glycolipid Deposition Protein D (Nicolaisen *et al.*, 2009a), an outer

membrane efflux channel that is responsible for exporting glycolipids from the periplasmic space to the cell exterior where they are deposited (Nicolaisen *et al.*, 2009a).

## Polysaccharide layer

The polysaccharide layer functions as a scaffold to support the glycolipid layer, protects the glycolipid layer form mechanical damage, and may serve to further limit oxygen diffusion into the cell (Figure 1.11). The polysaccharide layer is composed of repeating elements of one mannosyl and four glycosyl moieties linked by β(1-3)-glycosidic bonds with additional ß(1-4)-glycosidic glucose, xylose, galactose or mannose side-chains (Nicolaisen *et al.*, 2009a). HepA, HepB, and HepC have been implicated in the synthesis and assembly of the polysaccharide layer, however their specific functions have not been characterized (Adams, 2000).

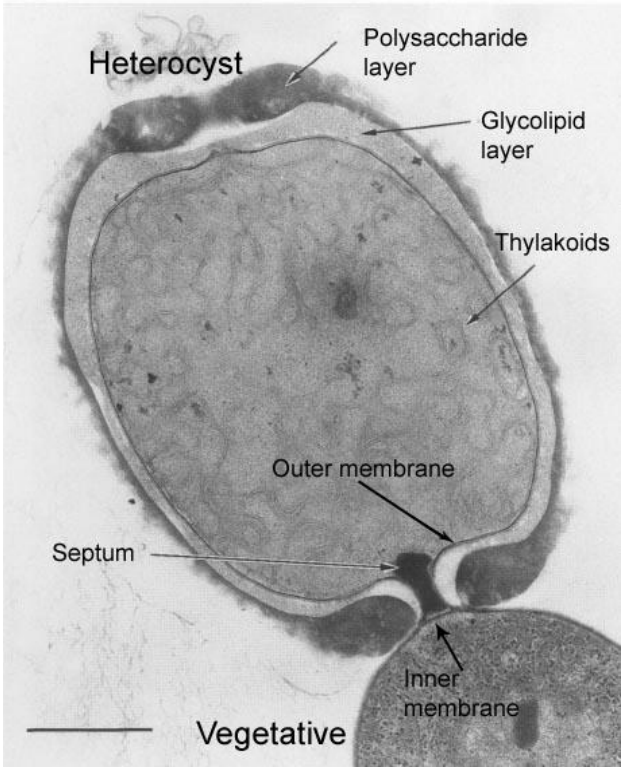



**Figure 1.11** Electron micrograph of a heterocyst demonstrating cell layers

http://www.moi.uni-tuebingen.de/research/images/anabaena.gif

## Biochemical changes in heterocysts

During the process of heterocyst differentiation, rapid degradation of many vegetative proteins occurs, coinciding with the synthesis of heterocyst-specific proteins as their replacement (Fleming and Haselkorn, 1974). This results in a fundamental reorganization of the metabolic machinery of the heterocyst that is geared toward protecting the nitrogenase from oxygen in these cells (Fleming and Haselkorn, 1974; Ownby *et al.*, 1979; Wood and Haselkorn, 1979). Heterocysts exhibit a loss in the ability to fix $CO_2$, a loss in $O_2$ evolution (Thomas, 1970), and a significant decrease in the light-harvesting proteins, phycobiliproteins and chlorophyll a (Tel-Or and Stewart,; Thomas, 1970). These finding are characteristic of the inactivation and degradation of photosystem II in heterocysts (Thomas, 1970), which eliminates oxygen evolution in the presence of nitrogenase. In contrast, an increase in photosystem I components is observed in heterocysts (Hawkesford *et al.*, 1983). PS I activity, which produces ATP by cyclic photophosphorylation in heterocysts, helps in meeting the energy needs of nitrogenase (Fay, 1970).

Another characteristic of heterocysts is that respiratory $O_2$ consumption is elevated three-fold in heterocysts compared to vegetative cells (Smith, 1985). Increased respiration serves to mop up any residual $O_2$ in the heterocyst and to supply energy to the nitrogenase in the form of ATP (Milligan *et al.*, 2007). Due to a loss in the ability to fix $CO_2$, heterocysts must import all fixed carbon from vegetative cells (Wolk, 1968). Heterocysts import fixed carbon in the form of a disaccharide, most likely sucrose (Cumino *et al.*, 2007; Porchia and Salerno, 1996; Schilling and Ehrnsperger, 1985). Heterocysts then convert the disaccharides into glucose-6-phosphate (Wolk *et al.*, 1995),

which is metabolized in the glycolytic pathway (Lex and Carr, 1974), and oxidized by the partial oxidative pentose phosphate pathway (Winkenbach and Wolk, 1973) to feed reductant into the nitrogenase complex (Wolk *et al.*, 1995).

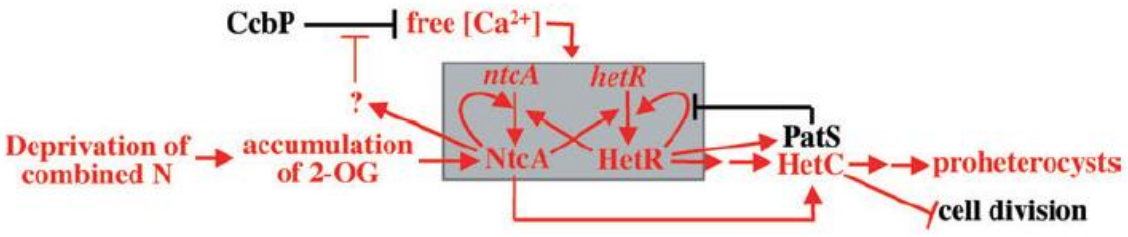
## Heterocyst Differentiation

Heterocyst differentiation is the process that metabolically and morphologically transforms a vegetative cell into a non-dividing cell whose only purpose is nitrogen fixation. Upon the removal of fixed nitrogen, many intercellular, extracellular, and intracellular signals must coordinate to bring about a functional heterocyst. Addition of extracellular fixed nitrogen sources prevents heterocyst differentiation. However, intracellular fixed nitrogen that is generated in all cells under anaerobic conditions by the *nif2* nitrogenase does not prevent heterocyst differentiation or pattern formation (Thiel and Pratte, 2001). This suggests that intracellular and extracellular nitrogen conditions are differentially sensed; thus the cells must recognize both intracellular and extracellular signals to trigger heterocyst differentiation and to rapidly shut down nitrogen fixation when fixed nitrogen becomes available.

Additionally, the cells must recognize intercellular signals in order to coordinate with adjacent cells to decide the developmental fate of each cell, which ultimately produces and maintains a regularly spaced pattern of heterocysts. The earliest of these signals includes calcium ions (Shi *et al.*, 2006; Torrecilla *et al.*, 2004; Zhao *et al.*, 2005), the Krebs cycle intermediate 2-OG (2-oxoglutarate) (Laurent *et al.*, 2005), and the transcriptional regulators NtcA and HetR (Zhang *et al.*, 2006). Cyanobacteria do not possess a complete Krebs cycle because they lack 2-OG dehydrogenase; thus, 2-OG is

used primarily by cyanobacteria as a carbon skeleton to assimilate fixed nitrogen via the glutamine synthetase–glutamate synthase cycle (Stewart, 1978) (Figure 1.3). Moreover, the intracellular 2-OG concentration varies as a function of nitrogen availability, and increased intracellular 2-OG concentration is perceived directly as nitrogen deficiency, which can trigger heterocyst differentiation in the presence of ammonium (Laurent *et al.*, 2005).

The sensor of 2-OG concentration is NtcA (Vazquez-Bermudez *et al.*, 2002), the global transcriptional regulator of nitrogen status. NtcA can act as an activator or repressor and its DNA-binding activity is stimulated in the presence of high 2-OG concentration (Vazquez-Bermudez *et al.*, 2002). NtcA activates it's own promoter, thus it is autoregulatory, and other promoters involved in nitrogen assimilation and heterocyst differentiation (Valladares *et al.*, 2008). One such promoter is that of *hetR*, the master regulator of heterocyst differentiation. HetR is a serine-type protease (Dong, 2000; Zhou *et al.*, 1998) that also acts as a transcriptional regulator (Khudyakov and Golden, 2004). HetR activates its own promoter, the promoter of *ntcA*, as well as many other promoters involved in heterocyst differentiation (Huang *et al.*, 2004). The expression of *hetR* and




(Zhang *et al.*, 2006)

**Figure 1.12** Master regulatory circuit for heterocyst differentiation.

*ntcA* are mutually dependent and create a dual circuit positive feedback loop (Figure 1.12) (Adams, 2000).

Another trigger of heterocyst differentiation is increased intracellular calcium concentration (Torrecilla, Leganes et al. 2004). Expression of an exogenous calcium storage protein was shown to prevent heterocyst differentiation, while mutation of the endogenous calcium storage protein, CcbP, lead to differentiation of multiple contiguous heterocysts (Torrecilla *et al.*, 2004; Zhao *et al.*, 2005). Normally, vegetative cells maintain low intracellular [$Ca^{2+}$]; however, in heterocysts NtcA and HetR participate in an additional positive feedback loop that increases [$Ca^{2+}$] (Shi *et al.*, 2006). Calcium ions stimulate the protease activity of HetR in heterocysts, which specifically degrades CcbP, the cyanobacterial calcium binding protein in these cells (Shi *et al.*, 2006). This releases stored calcium which further stimulates HetR to degrade more CcbP. Additionally, NtcA represses expression of *ccbP* dependent on 2-OG, which further raises the [Ca2+] in heterocysts (Shi *et al.*, 2006). Working together, NtcA and HetR cause a spike in intracellular calcium concentration in cells destined to become heterocysts, however it is unclear how calcium regulates heterocyst differentiation.

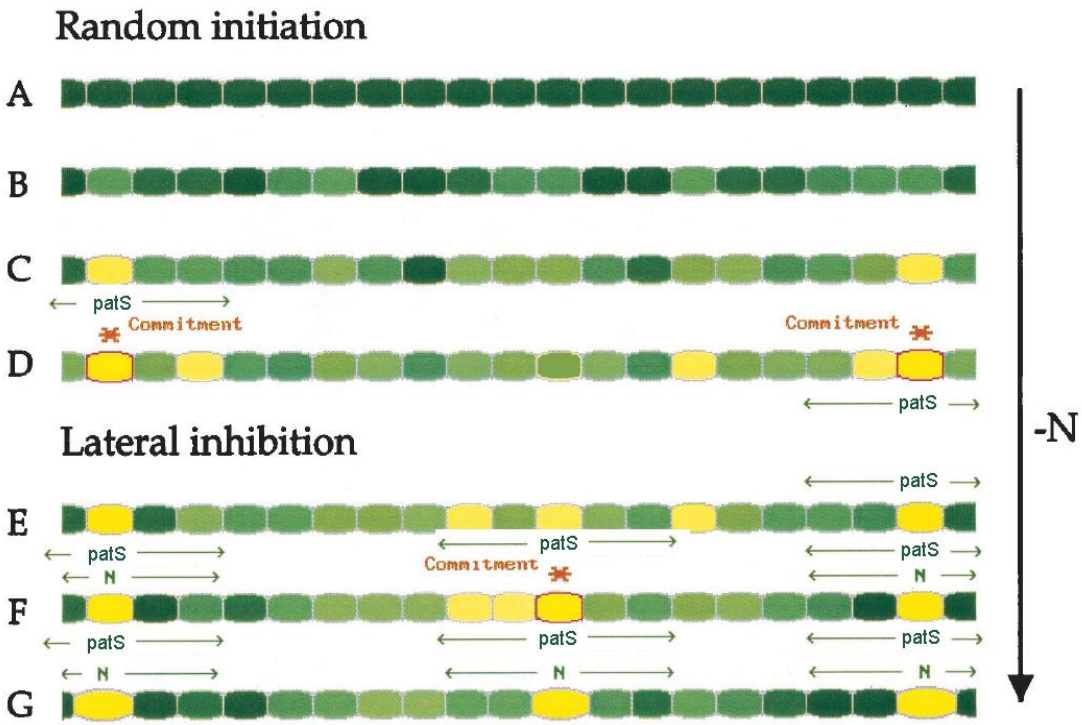
## Pattern formation

Upon removal of fixed nitrogen, individual cells randomly initiate the NtcA/HetR signaling cascade that leads to heterocyst differentiation depending on their specific nitrogen status. As the transition progresses, specific cells become irreversibly committed to differentiate, even if nitrogen is added back to the growth medium (Figure 1.13). At this time they are known as proheterocysts. Proheterocysts are associated with

the appearance of the cell envelope, the cessation of DNA replication, and the synthesis of PatS. PatS is the main gene product that is responsible for establishing the regular spacing of heterocysts; thus, PatS is considered the major protein governing pattern formation in *Anabaena* (Golden and Yoon, 2003). PatS acts as a diffusible inhibitor of heterocyst differentiation. PatS is made in developing heterocysts and diffuses into adjacent cells, which are at earlier stages of differentiation, causing them to regress back into vegetative cells (Wu *et al.*, 2004). This leads to resolution of clusters of differentiating cells into single heterocysts, producing a regularly-spaced pattern of heterocysts. The diffusion of PatS establishes regularly-spaced heterocysts because it forms a gradient in such a way that the cell midway between two heterocysts has the lowest concentration of the inhibitor, thus it is most likely to become a heterocyst (Figure 1.13) (Yoon and Golden, 2001). If PatS is the diffusible inhibitory signal of heterocyst differentiation, then HetR is the cytoplasmic receptor for this signal. PatS inhibits heterocyst differentiation by inhibiting the DNA binding activity of HetR, which prevents initiation of the signaling cascade for heterocyst differentiation (Huang *et al.*, 2004).

PatS functions to establish the initial pattern, while another patterning gene, *hetN*, functions to maintain the pattern. Borthakar *et al* (2005) demonstrated that a mutation in *patS* will initially develop randomly spaced or multiple contiguous heterocysts; however after a few days, a regular pattern is established and maintained (Borthakur *et al.*, 2005). In contrast, a *hetN* mutant will initially develop a regular pattern, but after a few days the pattern will disappear; resulting in multiple contiguous or misplaced heterocysts (Borthakur *et al.*, 2005). A mutation in both patterning genes results in death

because all cells eventually become heterocysts, which do not divide (Borthakur *et al.*, 2005; Orozco *et al.*, 2006).




Modified from Meeks *et al.*, 2002

Figure 1.13 Color indicates nitrogen status. Green to yellow represents high to low nitrogen. Individual cells randomly initiate heterocyst differentiation followed by lateral inhibition by PatS, which leads to resolution of single heterocysts that are evenly spaced.

# SECTION 2 : MATERIALS AND METHODS

## Strains and growth conditions.

Strains of *A. variabilis* FD, a derivative of *A. variabilis* 29413 that can grow at 40°C and supports the growth of bacteriophages better than the parent strain does (Currier and Wolk, 1979), and *Anabaena* sp. strain PCC 7120 were maintained on agar-solidified Allen and Arnon (AA) medium (Allen and Arnon, 1955) supplemented, when appropriate, with 5 mM $NH_4Cl$, 10 mM *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.2, 25 to 40 μg $ml^{-1}$ neomycin sulfate, or 3 μg $ml^{-1}$ each of spectinomycin and streptomycin. Strains were grown photoautotrophically in liquid cultures in an eightfold dilution of AA medium (AA/8) or in AA/8 supplemented with 5 mM $NH_4Cl$ and 10 mM TES, pH 7.2, at 30°C, with illumination at 50 to 80 microeinsteins $m^{-2}$ $s^{-1}$. Antibiotics were included as follows (when required): neomycin (3 to 5 μg $ml^{-1}$) and spectinomycin (0.3 μg $ml^{-1}$ for liquid). For experiments to measure the growth of strains with fructose, cells were harvested at an optical density at 720 nm ($OD_{720}$) of 0.2, washed once in AA/8, and resuspended in AA/8 with the indicated concentrations of fructose at an $OD_{720}$ of 0.02. All growth experiments were performed three times, and a representative graph is presented.

## Construction of plasmids and strains.

A neomycin resistance ($Nm^r$) cassette containing a transcriptional terminator was PCR amplified from pRL648 (Elhai and Wolk, 1988b), using primers nptTerm-3' and nptTerm-5', digested with *EcoR*I, and cloned into the *EcoR*I site of pUC1819RI to create

pBP285. Primer sequences are provided in Appendix 5.1. The $Nm^r$ cassette was used to create pBP299, a plasmid with an insertional mutation in *frtR*. The bom site of pRL1075, required for conjugation, was inserted into pBP299 to create pBP301. Replacement of the wild-type *frtR* gene in the chromosome of strain FD with the mutant *frtR* allele in pBP301 was accomplished by conjugation followed by double recombination (Elhai and Wolk, 1988a; Thiel and Wolk, 1987). The mutant was segregated as described previously and tested by PCR to verify that no wild-type copies of the gene remained (Lyons and Thiel, 1995). Plasmid pBP289 was created to contain the *ava2169* to *ava2173* genes from genomic library clone pAAWY3009. This plasmid was used to construct the replicating plasmids pBP291 (containing *frtRABC*) and pBP292 (containing *frt ABC* without *frtR*). Plasmids were constructed as described in Appendix 1. Replicating plasmids pBP291 (containing *frtRABC*) and pBP292 (containing *frtABC* without *frtR*) were conjugated into *Anabaena* sp. strain PCC 7120, selecting for the antibiotic resistance on the plasmid, and the presence of the plasmid was verified by PCR. The *Bgl*II and *Bam*HI $Nm^r$ fragment in pPE20 was replaced with a $Sp^r$ $Sm^r$ cassette containing a transcriptional terminator (amplified from pBP288, using the Sm - L and Sm - R primers, engineered with *Bgl*II and *Bam*HI sites at the 5' and 3' ends, respectively, for cloning) to create pBP350. The $Sp^r$ $Sm^r$ version of pPE20, pBP350, was used to create *lacZ* transcriptional fusions of *frtA* (pBP352) and *frtR* (pBP353) at the *Eco*RV and *Cla*I sites, respectively. Integration of the transcriptional fusions, pBP352 and pBP353, into the chromosome of FD was accomplished by conjugation of the nonreplicative plasmids, selecting for single recombinants containing the entire plasmid in the chromosome. Plasmid pBP288 is a 16.7-kb pBR322-based vector that contains (i) a promoterless *lacZ* gene for assaying

promoter activity in vivo; (ii) a 6.5-kb *ntcA* region of *A. variabilis* that allows for good homologous recombination; (iii) a 1.1-kb *npt* gene from pRL648 interrupting *ntcA*, ensuring only one functional copy of *ntcA* after recombination; (iv) a 1.0-kb $Sp^r$ $Sm^r$ cassette with a transcriptional terminator from pRL277 upstream of and directed away from the *lacZ* gene; and (v) a $Tet^r$ cassette between the *Bgl*II and *Sma*I cloning sites to allow for easy cloning of promoter fragments upstream of the *lacZ* gene. Plasmid pBP313 contained the *psbA* promoter in the *Bgl*II/*Sma*I sites of pBP288. A $Tet^r$ gene (PCR amplified from pBR322 by use of primers pBR322-L2 and pBR322-R2) was inserted into the *Sma*I/*Sac*I sites of pBP313 to create pBP328, a plasmid that destroyed the *lacZ* gene but gave selection for inserting fragments under the control of the *psbA* promoter in vivo. The plasmid used to overexpress FrtR in *A. variabilis* was constructed by PCR amplifying the *frtR* gene, using primers psbAFrtR-5 and psbAFrtR-3', and inserting it into the *Sma*I/*Sac*I sites of pBP328 to create pBP356. Additionally, the *frtABC* coding region (PCR amplified using primers frtABC-L and frtABC-R) was cloned downstream of the *psbA* promoter on pBP313 to generate pJU377. These plasmids were conjugated into FD by single recombination to yield BP356 and JU377. A 500-bp *frtA* promoter fragment (amplified from FD by use of the frtA498A-L and frtA-R10 primers) and a 400-bp *frtR* promoter fragment (amplified from FD by use of the frtR397-L and frtR-R10 primers) were cloned into the *Bgl*II/*Sma*I sites upstream of *lacZ* on pBP288 to generate pJU338 and pJU336, respectively. These plasmids were then conjugated, with selection for single recombinants, into FD to generate strains JU338 and JU336, into *Anabaena* sp. strain PCC 7120 to generate strains JU357 and JU356, and into BP301 to produce strains JU355 and JU353. The plasmid pBP354, used to overexpress FrtR in *Escherichia coli*,

was constructed by PCR amplification of *frtR* with *Nde*I and *Bam*HI sites at the 5' and 3' ends, respectively, using primers FrtR-L3 and FrtR-R3, and insertion into the same sites of pBP314. pBP314 was constructed by inserting a $Tet^r$ gene (PCR amplified from pBR322 by use of primers Tet(*Nde*I)-L and Tet(*Bam*HI)-R) into the *Nde*I/*Bam*HI sites of pET22b (Invitrogen), therefore making it easier to select for insertion of a DNA fragment encoding protein into the vector.

A 302-bp PCR fragment amplified from FD DNA using nifH302L/nifH1-R2 primers (Appendix 5.2) and cloned into the *Bgl*II/*Sma*I sites of pBP288 (Ungerer *et al.*, 2008) to produce pJU362. A 3.8-kb *Hind*III/*Sph*I fragment of pBR322 was cloned into the *Hind*III-*Sph*I sites in the pJU362 fragment containing *pnifH:lacZ* to yield pJU409. A $Nm^R$ cassette was amplified from pBP285 (Ungerer *et al.*, 2008) with primers nm5'termL/nm5'termR and cloned into the *Kpn*I site of pJU409 in an orientation opposite to *lacZ* to yield pJU410. The nm5'termL/nm5'termR primers incorporate a terminator at the 5' end of the $Nm^R$ cassette, oriented opposite to the $Nm^R$ cassette such that they terminate transcription at the 5' end of the $Nm^R$ cassette to prevent transcriptional read-through into *lacZ*. A 1.8-kb PCR fragment made from FD DNA using the primers frtB-L/frtB-R was then cloned into the *Sca*I/*Hind*III sites of pJU410 to yield pJU411. Other *lacZ* fusions were made by cloning various PCR fragment amplified from FD DNA into the *Bgl*II/*Sma*I sites in pJU411 as indicated in Appendix 5.1.

pJU445 was constructed by cloning a 9.5 kb fragment containing the *nif1* region (*ava3910- nifH1*) from pAAWY3162 (a plasmid made by JGI for sequencing the *A. variabilis* genome) into pBR322 using *Sal*I/*BamH*I. pJU445 was then digested with *Bsr*GI-*Msc*I (non-methylated MscI site), blunted and re-ligated to create a 3.1-kb deletion

of *nifB* and upstream sequences, producing strain pJU363. A 5.4-kb *Bgl*II fragment containing *sacB* and $Em^R$ from pRL2948a (Ungerer *et al.*, 2008) was cloned into the *Bam*HI site in pJU463 to produce pJU466. A 6-kb *nifS1-nifD1* fragment of pMV2 (Ungerer *et al.*, 2008) was cloned into pBR322 using *Bam*HI to produce pJU332. A 5-kb fragment of pPE20 (Thiel *et al.*, 1995) containing a promoterless *lacZ* was cloned into *nifH1* of pJU332 at the internal *Kpn*I site to yield pJU333.

Recombination of these plasmids into the *frt* region of *A. variabilis* by single crossover (Elhai and Wolk, 1988a; Thiel and Wolk, 1987) resulted in the strains with the same name as the plasmids (Appendix 5.2). Isolation of double recombinants was accomplished using *sacB* selection (Cai and Wolk, 1990; Gay *et al.*, 1985) on AA-$NH_4$ plates supplemented with 10% sucrose. Strains resulting from a single crossover in the *frt* region were identified by screening for colonies that were unable to grow in the dark with fructose. Strains resulting from a single crossover in the *nif1* region were screened for their $Nif^-$ phenotype by their decreased ability to grow on AA agar plates lacking a source of fixed nitrogen.

## FrtR overexpression, purification and electrophoretic mobility shift assay.

The FrtR protein was purified from *E. coli*/pBP354, overexpressing FrtR, as inclusion bodies as described by Campbell et al., with the following modifications: cells were lysed by four 30-s rounds of sonication and the protein concentration was adjusted to 1.0 mg $ml^{-1}$ before renaturation. Electrophoretic mobility shift assay binding reaction mixtures contained 4 mM Tris, pH 8.0, 12 mM HEPES, 12% glycerol, 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1.0 μg poly(dI-dC), and 10,000 cpm $^{32}$P-

end-labeled probe. FrtR was added (100 to 700 ng of protein), and the mixture was incubated for 20 min at 30°C. After the binding reaction, the reaction mixtures were loaded into a 4% polyacrylamide gel with a Tris-glycine buffer (pH 8.0) and were electrophoresed at 40 mA for 25 min. Bands were visualized using a phosphorimager.

**RNA isolation, RT-PCR, and 5' RACE.**

RNA was isolated from 50-ml cultures grown in AA/8 as follows. Cells were harvested, the media removed, and the cells were resuspended in 400 μl Tri Reagent (Sigma) and 200 mg of 150 micron glass beads was added. Cells were lysed via 2 sets of 60-second amalgamations using a Wig L Bug dental amalgamator followed by 60 seconds on ice. The Tri Reagent was removed to a new tube and the lysis step was repeated. The two aqueous phases were combined and extracted twice with chloroform. The RNA was then isopropanol precipitated and resuspended in 34 μl water + 1 μl RNasin (Promega). 10 μg of total nucleic acid was subjected to DNase digestion using a Turbo DNA-free kit (Ambion, Austin, TX). Reverse transcription-PCR (RT-PCR) was performed as previously described (Pratte and Thiel, 2006) as modified in (Ungerer *et al.*, 2008) with primers specific for each gene: *nifB* – nifBRTL/nifBRTR, *nifS* - nifSRTL/nifSRTR, *nifU* – nifU3'RTL/nifU3'RTR, *nifH* - nifHRTL/nifHRTR, and *nifK* - nifKRTL/nifKRTR.

For 5' RACE, 20 μg of RNA, treated with DNase, was extracted with phenol:chloroform:isoamyl alcohol, then with chloroform, followed by ethanol precipitation. The RNA was resuspended in 50 μl and half was treated with 20 U of Tobacco Acid Pyrophosphatase (TAP) (Epicentre, Madison, WI) for 60 min at 37° C.

The remaining half of the RNA was not treated with TAP but all subsequent treatments were performed on both samples. 200 pmol of the RNA adapter, RNAoligo09, was added to each tube. The RNA was extracted with phenol:chloroform:isoamyl alcohol, followed by extraction with chloroform, and ethanol precipitated. The pellet was resuspended in 14 μl water and ligated to the adapter using T4 ssRNA ligase (NEB) overnight at 17° C. The ligated RNA was extracted with organic solvents and ethanol precipitated as described above, resuspended in 20 μl water and reverse transcribed using Superscript III (Invitrogen) according to their protocol using the following primers *nifH* – nifHRTR, *nifU* – nifU3'RTR, *nifB* – nifBRTR2, *ava4055* – moe2-RPE2, and *vnfH*- vnfHPE1R. PCR was performed using the left primer oligoP1 and the following right primers: *nifH* – nifHout, *nifU* – nifURTR, *nifB* – nifBPCR1, *ava4055* – Moelike2-RPE, and *vnfH* – vnfHPE2R.

**Microtiter β-galactosidase assays.**

Cultures were grown as described above to an $OD_{720}$ of 0.1, divided into two equal portions, and 5 mM fructose was added to one portion to induce expression of the *frt* genes. Two hours after induction, the cultures were adjusted to an $OD_{720}$ of 0.05, and 700 μl of culture was added to 700 μl of 2x LacZ buffer (120 mM Na2HPO4, 80 mM $NaH_2PO_4$, 20 mM KCl, 2 mM $MgSO_4$, 100 mM β-mercaptoethanol). The samples were vortexed for 60 s with 30 μl 0.1% sodium dodecyl sulfate and 60 μl chloroform. The chloroform was removed, and 250 μl of sample was placed in microtiter wells. Eighty microliters of *o*-nitrophenyl-β-D-galactopyranoside (4 mg ml $^{1}$) was added to the wells,

and a microtiter plate reader measured the $OD_{420}$ every 90 s for 1 h. Eight replicates were done for each sample. Excel was used to process the raw data, yielding the rate of the reaction, which was normalized to the $OD_{720}$ of the culture.

**Standard β-galactosidase assay and acetylene reduction assays.**

For nitrogen stepdown experiments, cells grown in AA/8 supplemented with 5 mM $NH_4Cl$ and 10 mM TES at an $OD_{720}$ of 0.08-0.10 were washed 3 times with 25 ml AA/8 and resuspended at $OD_{720}$ of 0.025, without antibiotics. The cultures were split and 5 mM $NH_4Cl$, 10 mM TES, pH 7.2 was added to half. After 24 h the cells were harvested for the assay. β-galactosidase assays were performed as previously described (Miller, 1992). Acetylene reduction assay was performed as previously described (Pratte *et al.*, 2006; Shah *et al.*, 1975).

**Light micrographs.**

Filaments were viewed with a Zeiss epifluorescence microscope and imaged using a Retiga EXi (QImaging) cooled charge-coupled device camera with IP Labs 4.0 software (BD Biosciences). The exposure time was about 0.05 s for bright-field images.

# SECTION 3 : Regulation of nitrogenase genes in *A. variabilis*

Although much is known concerning the function of most of the *nif* gene products, very little is known about the transcriptional regulation of any of these genes in cyanobacteria. Moreover, no fragment of the *nifH1* upstream region has been found capable of driving expression of *nifH1* or a reporter. Transcription of the *nif* genes was first reported over 25 years ago (Haselkorn and Robinson, 1983; Jackman and Mulligan, 1995); however almost no progress has been made in identifying any aspects of transcriptional regulation.

A transcriptional start site for *nifH* has been identified at position -123 relative to the start codon in *Anabaena* sp. PCC 7120 (Haselkorn and Robinson, 1983; Jackman and Mulligan, 1995), while the transcription start site of *nifB* is -179 relative to its start codon (Mulligan and Haselkorn, 1989). Additionally, a non-canonical binding site for NtcA has been identified -40 to this transcription start site (Chastain *et al.*, 1990; Ramasubramanian *et al.*, 1994). NtcA was shown to bind a DNA fragment containing the *nifH1* promoter using a mobility shift assay, however the shift was weak and the authors did not use a non-specific competitor show the shift is specific (Chastain, 1990). Another potential transcriptional regulator of *nifH1* is FurA. FurA is the master regulator of iron status in cyanobacteria and is upregulated by NtcA specifically in heterocysts. These characteristics make FurA a good candidate for a regulator of *nifH1* because it is capable of regulating the nitrogenase (an iron rich protein) based on both iron and nitrogen status of the cell. In *Anabaena* PCC 7120 a potential binding site for FurA has also been identified in the *nifH1* upstream region. In this strain FurA binds across the *nifU* stop

codon in the intergenic region (Apendix 5.7) (López-Gomollón *et al.*, 2007). This site is similar in *A. variabilis* except that in *Anabaena* sp. PCC 7120 the 3' region of the binding site is composed of a pair of heptad direct repeats, while in *A. variabilis* the repeats are different and extend much farther into the intergenic region (Appendix 5.7). FurA is promiscuous in its recognition of target DNA sequences and has been shown to oligimerize along DNA (Lavrrar and McIntosh, 2003). I hypothesize that FurA may also regulate *nifH1* in *A. variabilis* by binding its target sequence and oligmerizing along the repeat sequence, which may also be recognized by FurA. Therefore, I investigated if the FurA binding site is required for expression of *nifH1* and whether the repeats also play a role in this process.

I have recently examined the region immediately upstream of *nifH1* in *A. variabilis* and have found that the intergenic region between *nifU1* and *nifH1* did not drive expression of *nifH1* or a *lacZ* reporter; thus I expanded the regions of analysis in order to identify those required for regulated expression of *nifHDK1* and its paralog, *vnfH*. I also reexamined the binding sites of the potential regulators NtcA and FurA using mutational analysis to determine their role in regulating *nifH1*.

# RESULTS

## Mutational analysis of nifH1 promoter and upstream sequences.

Previous attempts to identify a *nifH1* promoter fragment that could drive expression of *nifH1* or a reporter have failed, thus I decided to take an alternative approach to identify critical regulatory sequences. Large deletions and small 6 bp mutations of *nifH1* upstream sequences were constructed directly in the chromosome of *A. variabilis* (Figure 2.1). I then examined the mutant strains for loss of expression, or mis-regulation, of *nifH1*. The mutations were initially constructed in a plasmid bearing *nifH1* and upstream sequences. The mutant promoters were then transferred into a mutant strain that had a partial deletion of *nifH1* and its promoter, thus it was Nif$^-$.

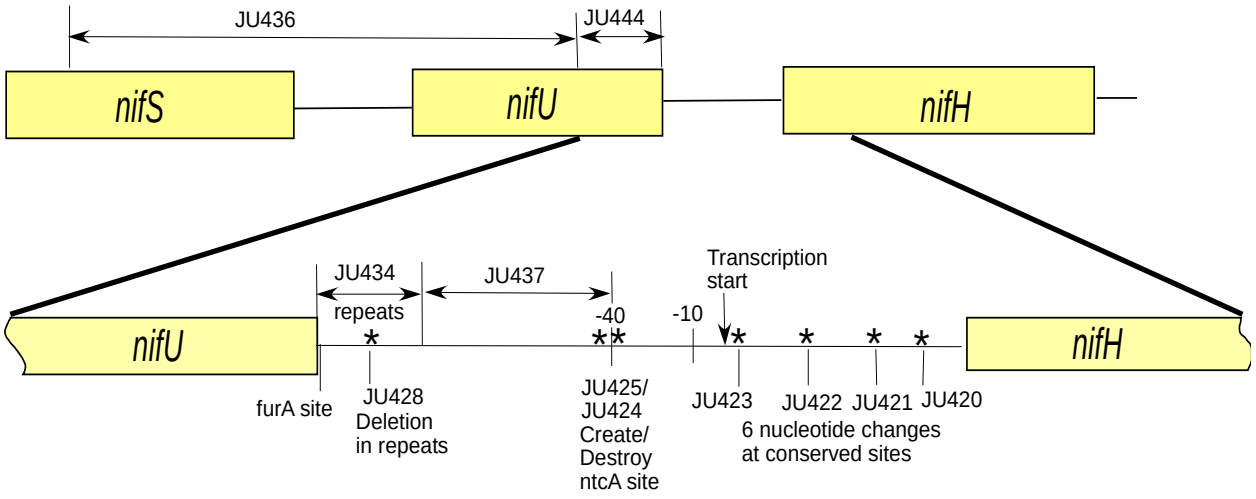



**Figure 3.1** – Diagram of chromosome deletions and mutations. Stars represent 6 bp mutations in the chromosome. Appendix 5.3 is an alignment showing specific location of the 6bp mutations. Larger deletions are represented as arrows spanning the region deleted.

Double recombination of the plasmid into this strain upstream and downstream of the promoter regions restore the chromosomal copy of *nifH1* with the mutant promoter, which allowed us to look for restoration of a $Nif^+$ phenotype and prevented complications of wild-type chromosomes being present, which would obscure our results.

The specific mutations that were made are as follows (see Figure 3.1): a pair of mutations in the proposed NtcA binding site, one to create a canonical NtcA binding site (JU424) and one to mutate the existing half-site (JU425), a small mutation of the repeat element located near the end of *nifU* (JU428), a deletion of the *furA* binding site located in the end of *nifU1* (López-Gomollón *et al.*, 2007)(JU434), a deletion of the -40 to the repeat element (JU437), a large deletion in the end of *nifU1* coding region (JU444), and a large deletion of the *nifSU1* coding region (JU436). Additionally, the *nifH1* promoters of 4 heterocyst forming cyanobacteria, *A. variabilis, Fischerella sp., Anabaena* sp. 7120, and *Anabaena L31* were aligned using Clustal to identify conserved sequences that would be targeted with further mutations (Appendix 5.3). I identified four short regions of conservation downstream of the transcriptional start site; however there was little similarity between the promoters upstream of the transcriptional start sites. A 6-bp mutation was made in each of the conserved sites to determine if they have a function.

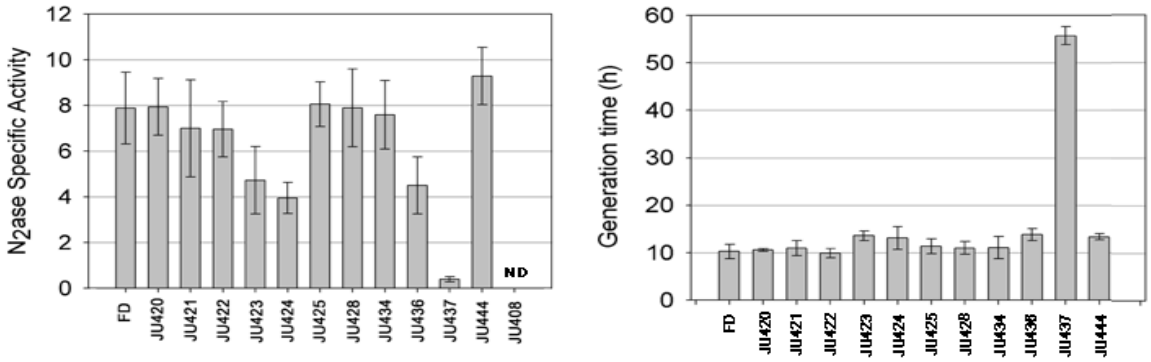



**Figure 3.2** – (A) Nitrogenase activity of *nif* promoter mutants. The parent strain JU408 showed no activity (ND). (B) Average generation time of *nif* mutants growing in the absence of nitrogen. Generation time is not shown for the parent strain JU408 because this strain failed to grow under the conditions tested.

None of the mutations in the conserved sequences had any effect on the ability of the strains to fix nitrogen, except for JU423, which is located at the putative transcription start site (Figure 3.2). The effect observed here is most likely due to its close proximity to the start of the transcript. Changing the *ntcA* site to create a canonical *ntcA* binding site reduced nitrogenase activity by half, while mutating the existing half-site had no effect on nitrogenase activity (Figure 3.2). This evidence suggests that NtcA does not activate *nifH1* by binding the -40 region as previously proposed. This is consistent with our own failure so show a specific shift with purified NtcA (unpublished data). A deletion of the FurA binding site or the repeat element also did not affect expression of *nifH1* (Figure 3.2).

A larger deletion extending from the -40 region upstream to the repeats did completely abolish nitrogenase expression (Figure 3.2); however promoter fragments extending through this region do not function. This suggests that while a functional promoter does not exist in the *nifUH1* intergenic region, sequences in this region are

necessary, but insufficient, to drive expression of *nifH1*. These results also suggest that there are additional sequences that are required for expression of *nifH1* that lie farther upstream or downstream of the *nifUH1* intergenic region.

A large deletion of the *nifSU* coding region reduced nitrogenase activity by half (Figure 3.2), where as mutation of either of these genes has been previously shown not to reduce nitrogenase activity (Lyons and Thiel, 1995). It is possible that the reduced nitrogenase activity was observed because the *nifSU* deletion inactivates two nitrogenase related genes; however I have identified a promoter element in the coding region for the *nifU* region (discussed below) and believe that the reduction in nitrogenase activity was a result of the loss of this promoter element.

## Sequences essential for *nifH1* or *vnfH* expression are far upstream from these genes

In order to identify regions upstream of *nifH1* that are essential for transcription, I constructed transcriptional fusions using DNA fragments of various sizes upstream from the *nifH1* gene to *lacZ*. These constructs were made in a plasmid that contained an internal fragment of the fructose transport operon (*frtBC*) (Ungerer *et al.*, 2008) for integration of the plasmid in the chromosome by single crossover (Figure 3.3). Depending on whether the crossover event between the plasmid and chromosome occurred in the *nifH1* promoter region or in the *frtBC* region, *lacZ* expression would be driven either by the entire normal chromosomal region upstream of *nifH1* (Figure 3.3B) or only the shorter *nifH1* upstream fragment (Figure 3.3A). Conversely, expression of the chromosomal copy of *nifHDK1* would be driven either by the normal upstream region

(Figure 3.3A) or the truncated plasmid-borne upstream region (Figure 3.3B). Measuring β-galactosidase or nitrogenase activity in strains in which expression of *lacZ* or *nifHDK1* was driven by promoter fragments of different sizes (Figure 3.3C) allowed us to determine the approximate location of the essential promoter elements.

I first examined strains containing the 300-bp fragment that comprised the entire *nifU1-nifH1* intergenic region fused to *lacZ* (Figure 3.3A). Strain JU457, in which the plasmid recombined into the *frtBC* region of homology (resulting in two defective *frtABC* operons) was identified by its $Frt^-$ phenotype (inability to grow heterotrophically in the dark with fructose). In JU457 the 300-bp *nifU1-nifH1* intergenic region fused to *lacZ* did not produce β-galactosidase (Figure 3.3A). The strain resulting from the alternative single crossover within the 300-bp *nifH1* upstream region, JU417, ($Frt^+$ and thus able to grow heterotrophically in the dark with fructose) provided all the *nifH1* upstream region driving expression of *lacZ*. JU417 gave β-galactosidase levels comparable to the positive control, JU333, in which a promoterless *lacZ* gene was inserted into *nifH1* in the chromosome by double crossover (Figure 3.3A). Strain JU417, in which expression of the chromosomal copy of *nifHDK1* was driven by the truncated upstream region ending at *nifU1* was unable to grow (data not shown) without a source of fixed nitrogen and lacked nitrogenase activity (Figure 3.4B). These data indicated that essential transcriptional elements were farther upstream than the 300-bp *nifU1-nifH1* intergenic region.

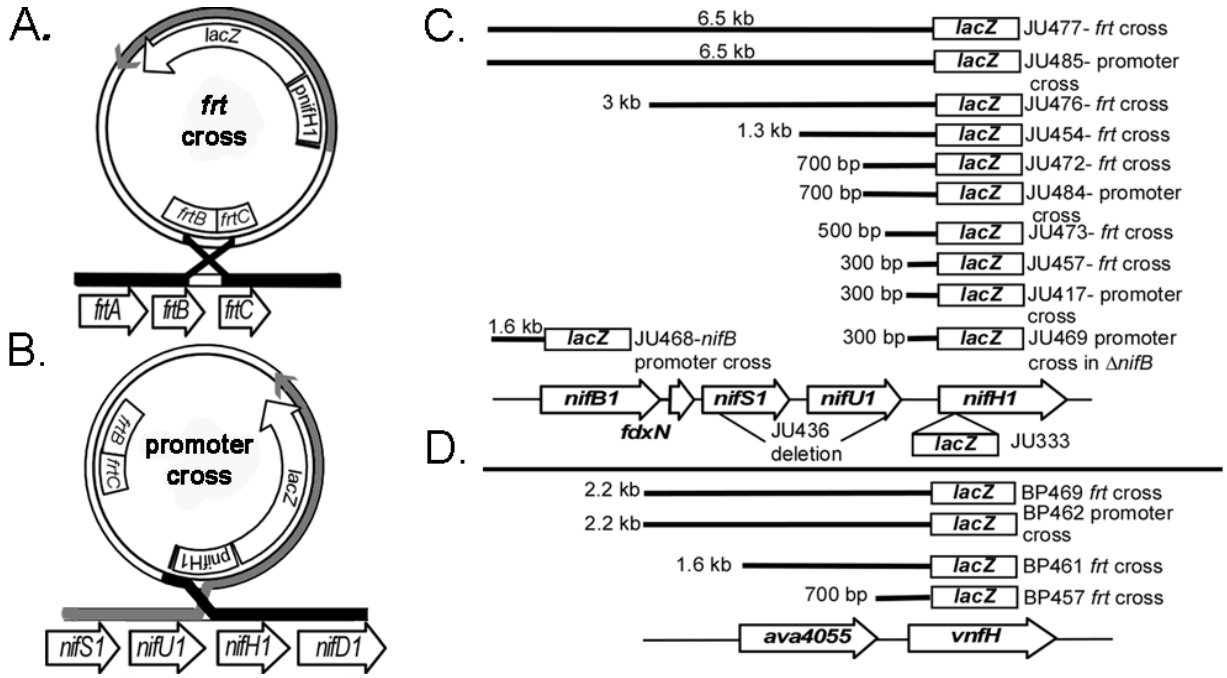



**Fig. 3.3.** Map of the genes analyzed or utilized in these studies. A and B. Two possible single-crossover events between plasmids bearing the promoter*:lacZ* fusions are shown: A. Recombination between the *frtBC* genes on the vector and in the chromosome resulted in a strain in which only the promoter fragment provided in the plasmid drove expression of *lacZ*. The chromosomal *nifHDK1* structural gene region was unchanged. B. Recombination between the *nifH1* promoter fragment in the plasmid and the chromosomal promoter placed *lacZ* under the control of the full, normal *nifH1* upstream region, including *nifBSU1*. The chromosomal *nifHDK1* structural genes are under the control of only the plasmid-borne promoter fragment. C. Diagram of the *nifH1* region with the strain names and sizes of the tested promoter fragments (not drawn to scale). Strain JU436 is a deletion of *nifSU* coding region as indicated. D. Diagram of the *vnfH* region with the strain names and sizes of the tested promoter fragments.

*A. variabilis* has an alternative V-nitrogenase, encoded by the *vnf* genes, that, like the *nif* genes, is repressed by fixed nitrogen and may share a similar mode of regulation with the principal Mo-nitrogenase (Pratte *et al.*, 2006; Thiel, 1993). We examined the intergenic region between *vnfH* and the upstream gene, *ava4055*, for its ability to drive expression of *lacZ* as described above for *nifH1*. In the strain in which the crossover occurred in the *frtBC* region, BP457, the *ava4055-vnfH* intergenic region did not drive *lacZ* expression. However, the strain with the alternative crossover within the *ava4055-vnfH* intergenic region, BP462, gave high levels of *lacZ* expression (Figure 3.5). These results indicated that essential promoter elements for *vnfH* lie upstream of the *ava4055-vnfH* intergenic region.

In order to identify the regions required for nitrogenase expression, plasmids with larger fragments extending farther upstream from *nifH1* or *vnfH* were constructed (Figure 3.3C, D). Strains in which these plasmids recombined using the *frtBC* region of homology, so that the truncated *nifH1* or *vnfH* upstream regions drove *lacZ* expression, were identified by their $Frt^-$ phenotype. JU473, containing a 500-bp *nifH1* upstream fragment that extended into *nifU1* did not drive expression of *lacZ*; however, JU472, with a 700-bp *nifH1* upstream fragment extending ~400bp into the *nifU1* coding region, provided ~25% of the β-galactosidase activity measured in strains in which *lacZ* expression was driven by the complete normal *nifH* upstream region (JU333 and JU417) (Figure 3.4A). Fragments of larger sizes (JU454, 1.3 kb; JU476, 3 kb) did not further increase β-galactosidase activity (Figure 3.4A). These results suggested that there was an essential transcriptional element in the 700-bp fragment (in strains JU472) that was missing in the 500-bp region (JU473), which placed it in the *nifU1* coding region. Strain

JU477, containing a 6.5-kb region, which extended from *nifH1* to 1.6 kb upstream of *nifB1*, provided levels of β-galactosidase activity similar to, but somewhat lower than, the strains in which *lacZ* expression was driven by the complete, normal *nifH* upstream region (JU333 and JU417) (Figure 3.4A.).

Strains in which recombination occurred in the promoter region of the plasmid were identified by their $Frt^+$ phenotype and verified by PCR (Figure 3.3B). Expression of the chromosomal copy of *nifHDK1* from the truncated promoter region was measured by nitrogenase activity. A 300-bp fragment (the *nifU1-nifH1* intergenic region) driving expression of *nifHDK1* gave no nitrogenase activity (Figure 3.4B), consistent with the results for *lacZ* expression from that same 300-bp fragment. A 3-kb fragment (extending from *nifH1* through *nifU1*) driving expression of *nifHDK1* (JU484) gave ~25% of the

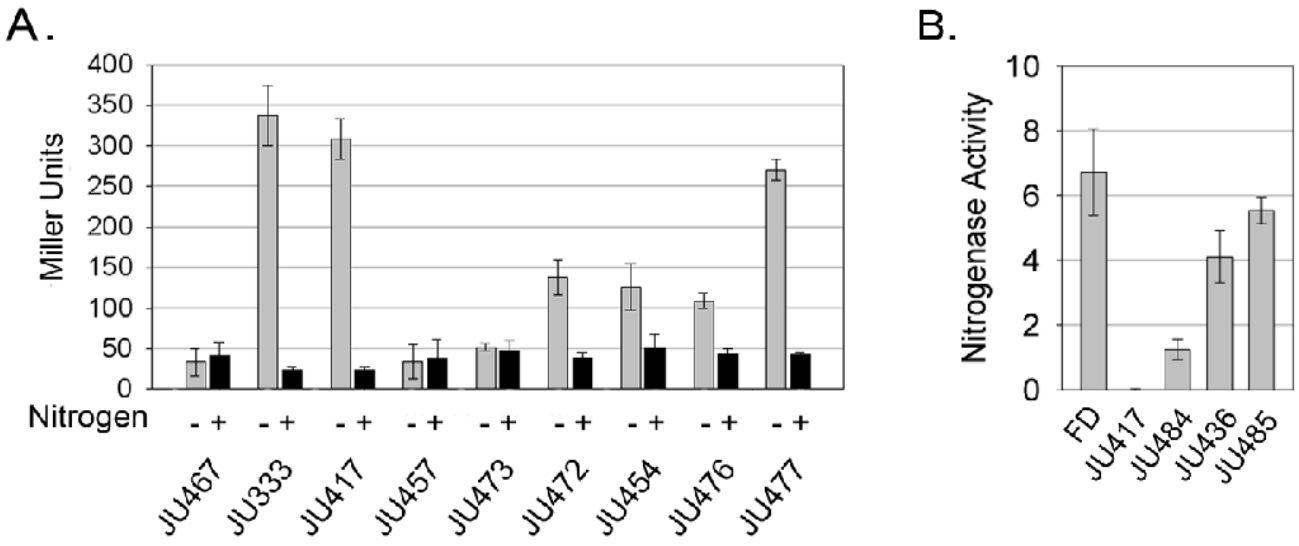



**Fig. 3.4** Expression from promoter regions. Expression of *lacZ*, by β-galactosidase activity of various sized promoter fragments (panels A), or nitrogenase activity, by acetylene reduction (panel B) from various promoter fragments or a *nifSU* deletion as shown in Fig. 3.3C. Nitrogenase is expressed as nmoles of ethylene mg $OD_{720}^{-1}$ $h^{-1}$. Strain JU467 is a promoterless *lacZ* fusion that is used to measure background.

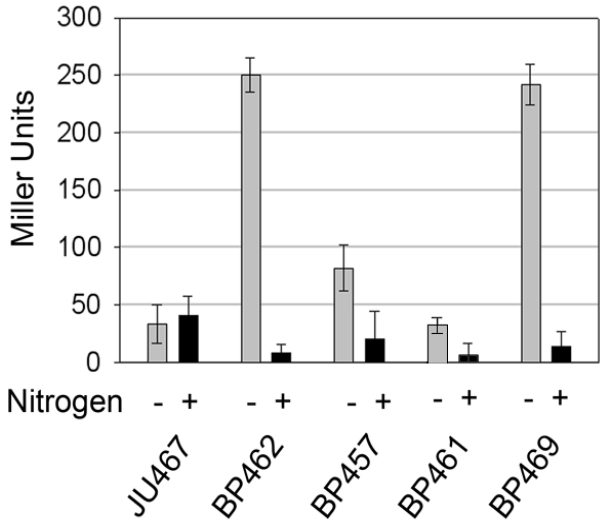



**Fig. 3.5**. Expression from *vnf* promoter regions. Expression of *lacZ*, by β-galactosidase activity of various sized promoter [1]. Strain JU467 is a promoterless *lacZ* fusion that is used to measure background.

Data kindly provided by Brenda Pratte

nitrogenase activity of the strain in which a 6.5-kb fragment (extending from *nifH1* to 1.6 kb upstream of *nifB1*) drove expression of *nifHDK1* (JU485) (Figure 3.4B). This was similar to the level of expression of *lacZ* driven by the 700-bp region that extended into *nifU1*; thus, together, these data suggested that there was a weak promoter in *nifU1*. The strain with a deletion of *nifS1-nifU1* (JU436), but with an otherwise complete wild-type upstream region, had ~75% of the nitrogenase activity of JU485 (Figure 3.4B). These data indicated that expression of *nifH1* required two regions; a weak promoter in the *nifU1* coding region and a strong promoter upstream of *fdxN*, possibly in the *nifB1* promoter region.

Essential sequences for the expression of *vnfH* were identified in the region upstream of *ava4055*. Two *vnfH* promoter fragments were constructed with the entire *ava4055* coding region. The first, BP461, extended only to the start of the *ava4055* coding region while the second, BP469, also included the *ava4055* promoter region (Figure 3.3D). Only BP469, which included the *ava4055* promoter region, had β-galactosidase activity that was comparable to the control strain, BP462, that had crossed

over in the *vnfH* region (Figure 3.5). Thus, the data indicated that the *ava4055* promoter drives expression of both itself and *vnfH*.

Previously published data indicated that *nifH1* and *vnfH* have their own promoters. Two pieces of supporting evidence have been published: 1) transcription start sites have been determined for *nifH* in other closely related cyanobacteria (Haselkorn and Robinson, 1983) and for *vnfH* (Pratte *et al.*, 2006), and 2) northern blots show a strong ~1.1 kb transcript corresponding to the size of the *nifH1* and *vnfH* genes alone (Pratte *et al.*, 2006). In the case of *nifHDK*, a stable transcript corresponding to the entire operon has also been reported (Mulligan and Haselkorn, 1989). However, the data shown in Figures 3.4 and 3.5 indicated that the intergenic regions between *nifU1* and *nifH1* and between *ava4055* and *vnfH* were unable to initiate transcription.

Together these finding suggest two possible hypotheses for *nifH1*: 1) Full transcriptional activation of *nifH1* requires upstream activation sequences; one activation element is in the *nifU1* coding region and the other element is shared with the *nifB1* promoter. In this model, transcription of *nifHDK1* originates from the putative transcriptional start site in the *nifU-nifH* intergenic region (Haselkorn and Robinson, 1983); however, the upstream activator elements work with this *nifH1* promoter to activate transcription at this start site and each upstream activator contributes to the expression. 2) Transcription does not originate at the previously identified *nifH1* transcription start site but rather from two separate, upstream promoters and continues into the *nifHDK1* operon. One promoter is in the *nifU1* coding region and the other is likely the *nifB1* promoter. The transcripts are then further processed in the intergenic region to produce discrete *nifBSU1* and *nifHDK1* transcripts.

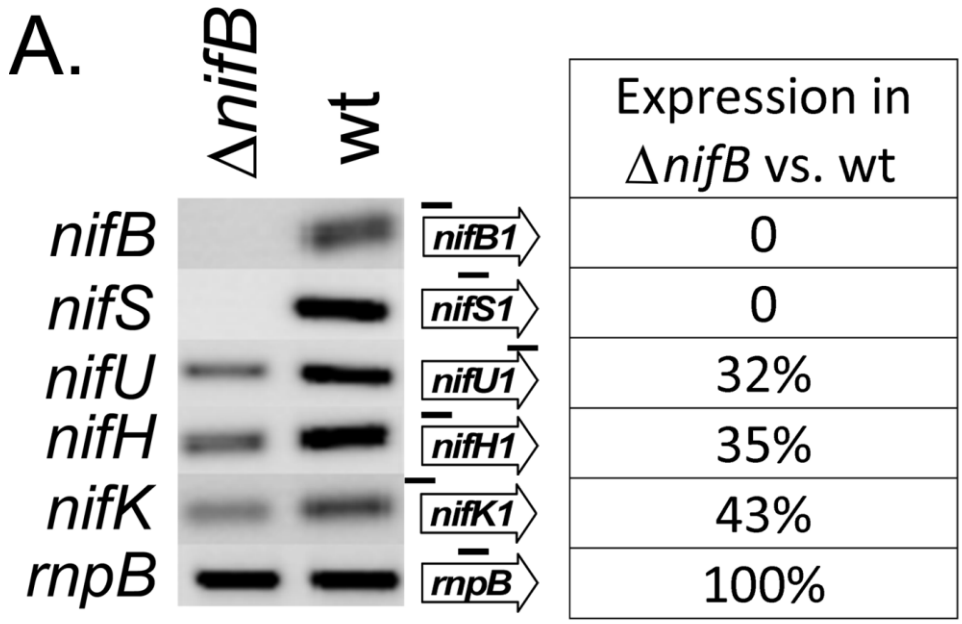



**Fig. 3.6** Expression of *nif1* genes in a *nifB1* deletion. A**.** RT-PCR of genes of the *nif1* cluster in the wild-type strain and in a *nifB1* promoter deletion strain. The genes amplified are shown next to the appropriate gel lanes. The black lines over the genes indicate the region amplified. The *rnpB* gene, which is constitutely expressed was used as a control to show equal amounts of RNA (Vioque, 1992).

## Identification of dual *nifH1* promoters

I have shown that a region upstream of *nifB1* is essential for high level expression of *nifH1*. If the *nifB1* promoter were required for expression of *nifH1* then I would expect a decrease in *nifHDK1* expression in a *nifB1* deletion mutant. Using semi-quantitative RT-PCR I measured expression of the *nif1* genes in a strain in which *nifB1* and its promoter were deleted. The *nifHDK1* genes were expressed in the *nifB1* mutant, although at a much lower level than in the wild-type strain (Figure 3.6). However, *nifU1*, which is believed to be under the control of the *nifB1* promoter and was shown by

northern blot analysis to be on the *nifBSU1* transcript (Mulligan and Haselkorn, 1989), was also expressed in the *nifB1* deletion (Figure 3.6). This indicated that there was an additional *nifU1* transcript originating from within *nifBSU1* and supports the hypothesis that the essential element in *nifU1* is a true promoter and is not an activator for a promoter in the *nifU1-nifH1* intergenic region. These results indicated that the *nifB1* promoter is required for high-level expression of *nifHDK1*. Thus it appears that expression of *nifHDK1* depends on dual promoters that initiate transcription upstream of *nifB1* and within *nifU1*.

The roles of the *nifB1* promoter and the internal *nifU1* promoter in expression of *nifH1* were examined separately. A plasmid with the 300-bp *nifU1-nifH1* intergenic region fused to *lacZ* was integrated into the chromosome at the *nifU1-nifH1* intergenic region in a *nifB1* deletion strain. This strain, JU469, had the *nifH1* upstream region, from the start of *nifH1* up to the end of *nifB1*, driving expression of *lacZ*; thus, it had the internal *nifU1* promoter but not the *nifB1* promoter. Expression of *lacZ* in JU469 was about one-third that of the control strain, JU417 (Figure 3.7). This level of expression was similar to expression from the 700-bp *nifH1* promoter fragment that contained only the promoter in *nifU1* (JU472). A plasmid with a 1.6-kb region upstream of *nifB1* fused to *lacZ* was integrated into the chromosome at the *nifB1* region to determine *lacZ* expression from the *nifB1* promoter alone. Expression in this strain, JU468, was about two-thirds of the control strain, JU417 (Figure 3.7). Moreover, the sum of the expression from the internal *nifU1* promoter and the *nifB1* promoter was similar to the level of expression of the control strain, JU417, which had both promoters. Thus, two promoters,

the *nifB1* promoter and a promoter within *nifU1*, are necessary and sufficient for expression of *nifHDK1* but the primary promoter for *nifHDK1* is the *nifB1* promoter.

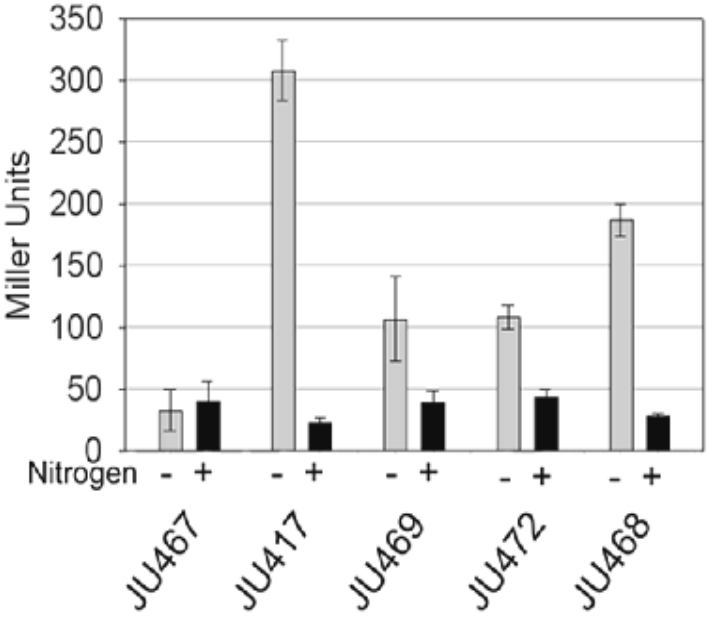



**Fig. 3.7.** Separate activity of the two *nifH1* promoters measured by β-galactosidase activity.

## Processing of the *nifBSUHDK* transcript

The *nifB1* promoter contributes substantially to the expression of *nifHDK1*; however, northern blot analysis indicates that in other cyanobacteria the *nifBSU* and *nifHDK* transcripts are separate (Jackman and Mulligan, 1995; Mulligan and Haselkorn, 1989; Ramirez *et al.*, 2005). This suggested that the larger *nifBSUHDK1* transcript may be efficiently cleaved post transcriptionally. I verified the apparent transcription start site of *nifH1* using 5' RACE, a method that can distinguish between processed and primary

transcripts (Bensing *et al.*, 1996). This technique requires the ligation of an RNA adapter to the 5' end of the transcript. The ligation is impaired by the 5' triphosphate present on primary transcripts; thus, ligation requires treatment of the sample with tobacco acid phosphatase (TAP), which hydrolyzes the triphosphate to a monophosphate. Processed transcripts already have a 5' monophosphate, thus TAP is not required for ligation of a processed transcript to the adapter. If the ligation reaction works equally well with or without TAP then the transcript is processed. I performed RNA ligase-mediated RT-PCR and then recovered and sequenced the cDNA bands to determine the *nifH1*, *nifB1*, and internal *nifU1* transcription start sites. The transcription start site of *nifH1*, as determined by 5' RACE, was within a few nucleotides of the previously determined *nifH* start sites for *Anabaena* sp. 7120 (Haselkorn and Robinson, 1983) and *Anabaena azollae* (Jackman and Mulligan, 1995); however the same product was made in RNA samples treated with or without TAP (Figure 3.8). This indicated that the putative transcription start site in the *nifU1-nifH1* intergenic region is actually a site at which the larger transcript is processed rather than a transcription start site. The transcription start sites identified in *nifU1* and upstream of *nifB1* were primary transcript start sites, since the reactions gave a strong product only after treatment of the RNA with TAP (Figure 3.8).

Based on sequencing of the *nifU1* 5' RACE reaction product, the transcriptional start site in *nifU1* is 320 nucleotides upstream from the 3' end of *nifU1*, within the 700-bp promoter fragment, which was the smallest promoter fragment that drove expression of *lacZ*. I identified a pair of direct repeats, GCGGTT, -35 to this transcriptional start site that might serve as a binding site for a regulator or heterocyst-specific sigma factor (Figure 3.8B). The sequence of the 5' RACE reaction product for *nifB1* placed the

transcription start site within a few nucleotides of the published transcription start site for *nifB* in *A. azollae*, a strain whose sequence in the entire *nif* region is identical to *A. variabilis* (Mulligan and Haselkorn, 1989). Alignment of the *nifB1* and *nifU1* promoters yielded no significant similarities between the two *nif1* promoters, suggesting that the two promoters do not share a similar mode of regulation.

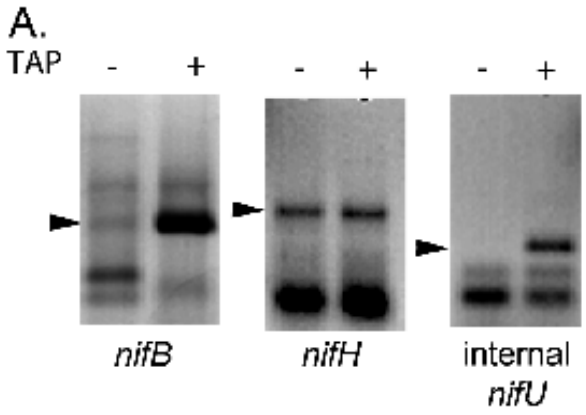



**Fig. 3.8.** 5' RACE to determine transcripts beginning upstream of: A. *nifB1*, *nifH1*, and an internal region of *nifU1*; and B. The transcription start site in *nifU1*, which is located 320 nucleotides upstream of the 3' end of *nifU1*. Arrows indicate the products that were sequenced

B. **Transcriptional start site (tss) in *nifU***

```
      -35(direct repeat)          -10            *tss                nifU stop
TGGCGGTTGCGGTTCCTGTTTAGCTAAAATTGATGATATCATTAAAGATGTAAAGGAAAA//…310bp…//TAG
```

## Processing of the *ava4055-vnfH* transcript

The expression of *vnfH* was shown to require only the *ava4055* promoter; however, *vnfH* and *ava4055* are found on separate transcripts (Pratte *et al.*, 2006), suggesting that the apparent *vnfH* transcript may also result from processing. The bands obtained by 5' RACE, using RNA with or without TAP, for the *vnfH* transcript were of

equal intensity, indicating that *vnfH* is a processed transcript (Figure 3.9). The sequence of the RNA ligase-mediated RT-PCR product revealed a start site within a few nucleotides of the transcription start site that was identified by primer extension (Pratte *et al.*, 2006). Amplification of the *ava4055* transcript by 5' RACE, using RNA either treated with or without TAP, yielded a product only when the RNA was treated with TAP, indicating that it is a primary transcript (Figure 3.9).

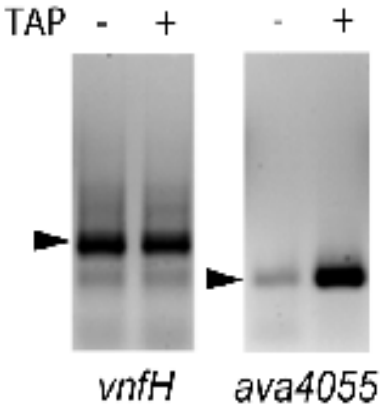



**Fig 3.9** 5' RACE to determine transcripts beginning upstream of *vnfH* and *ava4055*. Arrows indicate the products that were sequenced.

# Discussion

Since the initial identification of the *nifH* transcription start site almost three decades ago (Haselkorn and Robinson, 1983; Jackman and Mulligan, 1995), little progress has been made in identifying the sites or factors that lead to heterocyst-specific, nitrogen-regulated expression of *nifHDK* in cyanobacteria. Our attempts to identify any fragment in the *nifU1-nifH1* intergenic region of *A. variabilis* that could drive wild-type levels of expression of *nifH1* or a *lacZ* reporter were unsuccessful. I demonstrated here that the reasons are two-fold. First and foremost, the *nifHDK1* transcript is actually a cleavage product of a larger transcript, thus it does not have its own promoter. Secondly, normal expression of *nifHDK1* is a result of at least two promoters that function together to provide high-level expression of the nitrogenase structural genes. The transcript is then cleaved in the *nifU1-nifH1* intergenic region to produce two distinct mRNAs. Cleavage of the *nifBSUHDK1* transcript is likely to be very efficient, perhaps co-transcriptionally, as it is difficult to detect the full length transcript by northern analysis (Golden *et al.*, 1991; Ramirez *et al.*, 2005). The processing of polycistronic mRNAs is a common method of post-transcriptional regulation that is commonly employed by bacteria to allow coordinated expression of several genes from a single promoter while providing non-stoichiometric expression of individual genes of the operon (Grunberg-Manago, 1999; Newbury *et al.*, 1987).

The differential stability of transcript segments is a result of stem-loop structures at the extreme 5' or 3' ends of the mRNA (Grunberg-Manago, 1999). These structures have been reported to stabilize specific regions of a transcript relative to the whole (Sawers, 2006). In order to afford stability to an mRNA, a stem-loop must be positioned

no more than two nucleotides from the 5' or 3' end (Emory *et al.*, 1992). The initial cleavage of a polycistronic mRNA often occurs in an intercistronic region and is often mediated by RNaseE (Alifano *et al.*, 1994). RNaseP then trims the 5' leader to the base of a stem-loop, a position at which the stem-loop can protect the mRNA from degradation by acting to block the initiation of degradation (Alifano *et al.*, 1994; Li and Altman, 2003). In *E. coli*, an otherwise unstable transcript can be stabilized by fusing a stem-loop structure to the extreme 5' end of the transcript (Alifano *et al.*, 1994). Stem-loop structures have also been observed to act as degradation barriers when present at the 3' end of the transcript. In *Rhodobacter capsulatas* the photosynthetic genes *pufBALMX* are arranged on a single operon; however the half-life of the *pufLMX* segment is three minutes while the half-life of the *pufBA* segment is 20 minutes, resulting in large differences in protein expression from the two transcript segments (Chen *et al.*, 1988). In this case, an intercistronic stem-loop at the 3' end of *pufBA* acts as a decay terminator that prevents degradation of the *pufLMX* segment from extending into the *pufBA* genes.

The putative transcription start site originally mapped to the intergenic region upstream of *nifH* (Haselkorn and Robinson, 1983; Jackman and Mulligan, 1995) does not result from the initiation of transcription. This explains the failure to identify the key regulatory regions controlling *nifHDK1* expression. The data presented here show that the *nifBSUHDK1* genes are co-regulated under the control of the *nifB1* promoter and the internal *nifU1* promoter; however the contribution to *nifHDK1* expression from the two promoters is not equal**.** The *nifB1* promoter was responsible for 70-75% of the *nifHDK1* transcript while the internal *nifU1* promoter produced about 25-30%, as evidenced by reporter expression from strains containing either *nifB1* (JU468) or internal *nifU1*

(JU469) promoters. Furthermore, when the *nifHDK1* genes were expressed from only the internal *nifU1* promoter (JU484), nitrogenase activity was 25% of the level observed when both promoters drove expression of these genes. When the *nifHDK1* genes were expressed from only the *nifB1* promoter (JU436) in a *nifU1-nifS1* deletion strain, nitrogenase activity was 75% of the level observed when both promoters drove expression of these genes. It was shown previously that neither NifS1 nor NifU1 is required for nitrogenase activity, presumably because other proteins, perhaps those that make Fe-S clusters for photosynthesis, function in their place (Lyons and Thiel, 1995). Together these findings suggest that the *nifB1* promoter is the primary promoter driving expression of *nifHDK1*.

Microarray data indicate that the *nifHD* genes of *Anabaena* sp. 7120 are expressed more strongly than the *nifBSU* genes (Ehira *et al.*, 2003). This is due, at least in part, to the second promoter in *nifU*. However, the low activity from the internal *nifU1* promoter in *A. variabilis* would be insufficient to account for the large difference in expression between the *nifBSU* and *nifHDK* operons observed in *Anabaena* sp. 7120 (Ehira *et al.*, 2003). Therefore, I hypothesize that RNA processing could also contribute to increased expression of *nifHDK* relative to *nifBSU*. If a processing event places a stem-loop structure at the 5' end of the transcript then that transcript will have increased stability (Alifano *et al.*, 1994). Processing of a *nifHDK* transcript in *Rhodobacter* (Willison *et al.*, 1993) and of a *nifH* transcript in *Heliobacter* (Enkh-Amgalan *et al.*, 2006) at the base of a stem-loop structure has been reported and there appears to be processing of *nifHDK* transcripts in *Trichodesmium* as well (Dominic *et al.*, 1998). I investigated the 5'UTR of the *nifH1* and *vnfH* genes for potential secondary structure

close to the 5’ end of the transcript. The 5’UTRs of both *nifH1* and *vnfH* can potentially fold into very similar secondary structures and they share some sequence identity, particularly in the folded region (Figure 3.10). The base of the first stem is conserved, except that the fourth and sixth nucleotides of the stem are different between *nifH1* and *vnfH*; however, evolution has created compensating mutations in the stem to retain base pairing, which further supports a function for the structure. Moreover, the position of the 5’ end of the transcript at the base of the first stem is conserved for both genes, and this is the specific position that is required for a 5’ hairpin structure to afford stability to the transcript (Alifano *et al.*, 1994) (Figure 3.10). This suggests that processing of the transcript at the stem could provide additional stability to the *nifHDK1* and *vnfH* segments of the transcript. The additional stability of the *nifHDK1* segment relative to the *nifBSU1* segment may provide the proper ratio of nitrogenase proteins that is required for

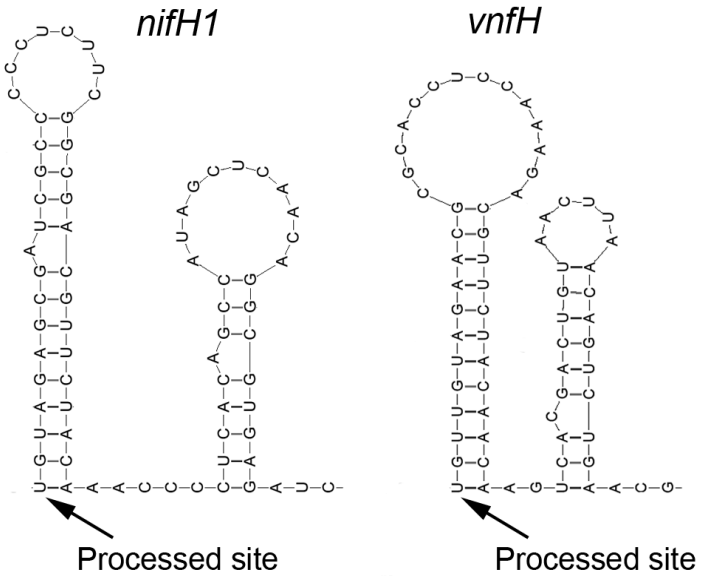



**Fig. 3.10** Predicted secondary structure near the processed 5’ start sites of the *nifH1* and *vnfH* transcripts predicted using mfold.

nitrogen fixation. These findings suggest that processing of *nif* transcripts may be a common mode of gene regulation in cyanobacteria.

Suzuki *et al*. found evidence that *nifBSU* and *nifHDK* are co-regulated in *Anabaena* sp. 7120. Mutants that lost the transcriptional activator AnCrpA, which was shown to bind to the *nifB* promoter, but not *nifH*, showed decreased expression of both the *nifBSU* and *nifHDK* operons (Suzuki *et al.*, 2007). Thus, the decreased expression of *nifHDK* may result from decreased *nifB* promoter activity in this *ancrpA* mutant. In addition to the *nifB* promoter, I have also identified a promoter in the *nifU* coding region. Although this promoter cannot drive high-level expression of *nifHDK*, it likely contributes to the increased level of expression of *nifHDK* relative to *nifBSU*. Several genes exhibiting dual promoters have been identified recently in *Anabaena* sp. 7120, such as *devB, hetR*, *hetC,* and *coxBAC* (Muro-Pastor *et al.*, 2009; Schmetterer *et al.*, 2001). In fact, the *coxBAC* operon of *A. variabilis* utilizes dual promoters and processing of the mRNA to achieve proper regulation (Schmetterer *et al.*, 2001). The *zwf* operon of *Nostoc punctiforme* is another interesting example of multiple promoters. This operon has four genes on a transcript; however, there are additional promoters internal to the operon that are differentially regulated depending on the carbon and nitrogen sources (Summers and Meeks, 1996). In the case of *nifH1*, dual promoters are used to produce higher levels of expression than can be achieved using a single promoter. This is similar to the *coxBAC* operon in that both promoters must be functioning simultaneously to provide the maximum level of expression (Schmetterer, Valladares et al. 2001).

Development requires complex changes in gene expression and precise, coordinated timing of gene expression. Multicellular organisms utilize multiple

promoters to allow for different levels of expression of the same gene in different cell types, during various stages of development, or under different environmental conditions (reviewed in Schibler and Sierra, 1987). The leader sequences of multiple transcripts can significantly affect the stability leading to differences in gene expression (Emory *et al.*, 1992). *Anabaena* is a model organism for the study of development and the origins of multicellularity. Recently, several key developmental regulators of heterocyst differentiation have been found to have multiple promoters including *ntcA, hetR*, and *hetC* (Muro-Pastor *et al.*, 2009; Ramasubramanian *et al.*, 1996). Also, genes that are differentially expressed between vegetative cells and heterocysts, such as *glnA, petH* and *ntcA*, have been shown to accomplish this through the use of multiple promoters (Ramasubramanian *et al.*, 1996; Tumer *et al.*, 1983; Valladares *et al.*, 1999). Data presented here indicate that the *nifHDK1* operon is also controlled by multiple promoters. This suggests that the use of multiple promoters to coordinate changes in gene expression during development may be common to organisms that undergo cellular development.

# Future Directions

## Secondary structure on the *nifHDK* transcript

I hypothesize that the step-loop structure in the 5' end of the *nifHDK* transcript serves to stabilize this segment of a larger transcript; however direct evident for this effect is lacking. Two approaches will be used to verify this hypothesis. First we will demonstrate that this structure can stabilize another transcript in *E. coli*. Previous work in *E. coli* indicated that fusion of a stabilizing step-loop to the extreme 5' end of an otherwise unstable transcript can stabilize that transcript (Bricker and Belasco, 1999; Emory *et al.*, 1992). We will verify that the stem-loop on the 5' end of the *nifH* transcript has a stabilizing effect by fusing this region onto the 5' end of a transcript containing the reporter *lacZ*. This strain will be constructed in a way such that nucleotide at the base of the first stem-loop is the same nucleotide at which the *lac* promoter initiates transcription; thus the stem-loop structure will be on the extreme 5' end of its transcript. We will also construct a similar strain in which the same stem-loop is 10 nucleotides internal to the transcript, a position at which the structure can no longer stabilize the transcript. β-galactosidase assays will then be used to compare the unmodified *lac* promoter to the two that have stem-loops in their 5' ends. If the stem-loop does stabilize the transcript then we will observe increased β-galactosidase activity in the strain containing this structure at the extreme 5' end of its transcript; but not when this same structure is 10 bp internal to the transcript or not present.

Second, we will determine the effects of the stem-loop on expression of the *nifHDK* transcript in *A. variabilis*. Ten-bp mutations will be constructed in the chromosome of *A. variabilis*, one to disrupt base pairing in each of the stem-loop structures (Figure 3.10), as well as a larger mutation that disrupts both stem-loop structures. We will also construct compensating mutations that restore base pairing in the mutant strains. Nitrogenase activity and growth rate will be determined for the mutant strains to verify that disrupting the stem-loop structures affects nitrogenase activity. If the stem-loop structure does serve to stabilize the transcript, then we expect to observe decreased nitrogenase expression and growth in the strains with mutations that disrupt the stem-loops; while strains with compensating mutations will exhibit nitrogenase activity and growth rates comparable to wild type.

## Secondary structure in the *nifU-nifH* intergenic region

The mutations and deletions from the chromosome suggest that two regions between *nifU1* and *nifH1* are important for expression of *nifH1*. The first region is near the processing site between *nifU1* and *nifH1* (Figure 3.2). Mutations in this region were able to reduce expression of the nitrogenase by half (JU424), presumably by disrupting the stabilizing structure or processing event at the 5' end of the *nifH1* transcript. The second region, which abolished expression of *nifH1* when mutated (JU437) (Figure 3.2) (Appendix 5.4, pink overline), is the region between the *nifH1* processing site and *nifU1* stop codon. Interestingly, this region contains the only sequences in the *nifU1-nifH1* intergenic region that are not conserved with the closely related species, *Anabaena* sp.

7120 (Appendix 5.4, pink overline). In fact, most of this region is not present in *Anabaena* sp. 7120 as the *nifU1-nifH1* intergenic region is much shorter in this species. This raised the question of whether this region, although much shorter in *Anabaena* sp. 7120, is also necessary for expression of *nifH1* in this strain because *A. variabilis* and *Anabaena* sp. 7120 are believed to regulate *nifH1* in a similar manner. This can be confirmed by deleting this same region from the chromosome of *Anabaena* sp. 7120 and determining if this mutant is also impaired in nitrogen fixation using acetylene reduction or growth assays. If this region is also essential for expression of *nifH* in *Anabaena* sp. 7120 we be able to significantly narrow down the sequences that are essential for expression of *nifH1* in *A. variabilis* because the sequences that are conserved between the strains are likely to be those essential for expression of *nifH* and most of this region is missing in *Anabaena* sp. 7120 (Appendix 5.4).

The sequence of the essential region is not conserved between *Anabaena* sp. 7120 and *A. variabilis*; however the ability of the two regions to fold into a stem-loop is conserved (Appendix 5.8). This suggests that the structure rather than sequence between the *nifU1* stop codon and the *nifH1* processed site is essential for expression of *nifH1*. In *A. variabilis*, this region is capable of forming three stem-loop structures (Figure 3.11), one large stem loop can form from the whole region (B), while two shorter stem-loop structures can form from the 5' and 3' halves of this region (A and C). This configuration is reminiscent of terminator-antiterminator structures that are commonly found in bacteria. In this model the first stem-loop (A) would function as a terminator, while stem-loop (B) would function as an antiterminator by preventing formation of stem-loops (A) and (C). The function of stem-loop (C) is to prevent formation of stem-

loop (B), which has the effect of stabilizing stem-loop (A). The chromosomal deletions in the *nifU1-nifH1* intergenic region indicate that a deletion (JU444) that disrupts the larger structure (B) or the 5' structure (A) does not affect nitrogenase expression (Figure 3.2) (Appendix 5.4, green overline), however a larger deletion (JU437) (Appendix 5.4, pink overline) (Figure 3.2) that disrupts the smaller 3' structure and deletes additional sequences that are not present in *Anabaena* sp. 7120 completely abolishes nitrogenase activity. The mutation that abolishes nitrogenase activity destroys the large stem loop (B) and the smaller one that can form from the 3' of the region (C). The mutation that does not affect activity abolished the large stem-loop (B) and the smaller structure that forms at the 5' of the region (A). This data is consistent with these structures forming a terminator/antiterminator structure as a deletion of the hypothetical terminator structure (A) had an effect on expression, while a mutation that prevents the anti-terminator (B and C) from forming abolished nitrogenase expression; presumably by allowing formation of only the terminator, which terminates transcription before it reaches the structural genes.

We will perform mutational analysis on this region and attempt to ascribe a function to each of the potential secondary structures. First we will generate a more specific chromosomal mutation that disrupts only the 3' stem-loop (C) without deleting downstream sequences. This mutation is necessary to verify that a disruption of the stem-loop, and not a loss of other sequences prevented nitrogenase expression in JU437. Mutation of stem-loop (A) in conjunction with (B) (JU428) was already shown not to affect nitrogenase activity (Figure 3.2). Assuming expression of nitrogenase is dependant on stem-loop (B), we will continue by making several additional mutations and compensating mutations that will allow us to separate the functions of each of the three

structures. We will make two compensating mutations, one to restore stem-loop (A) and one to restore stem-loop (C) in the mutants that have disrupted base pairing of these stem-loops (JU428 and the stem-loop C mutant discussed above). It is important to note that neither compensating mutation will restore stem-loop (B); thus another compensating mutation that restores the base pairing of stem-loop (B), while preventing base pairing of stem-loops (A) and (C) will also be made.

These mutations will allow us to separate the individual functions of each stem-loop because we will have mutated each one individually or in combination with another stem-loop. If the larger stem loop (B) functions as an anti-terminator then we expect that disrupting this structure will allow a *nifU* terminator to always form. This will prevent expression of the nitrogenase unless the 5' stem loop (A), which may function as a terminator is also mutated. A compensating mutation that restores the ability to form the larger stem-loop will restore nitrogenase activity as the anti-terminator stem-loop can now prevent formation of the *nifU* terminator. Any mutation that disrupts stem-loop (A) will not affect nitrogenase expression because these mutations will always prevent the terminator from forming.

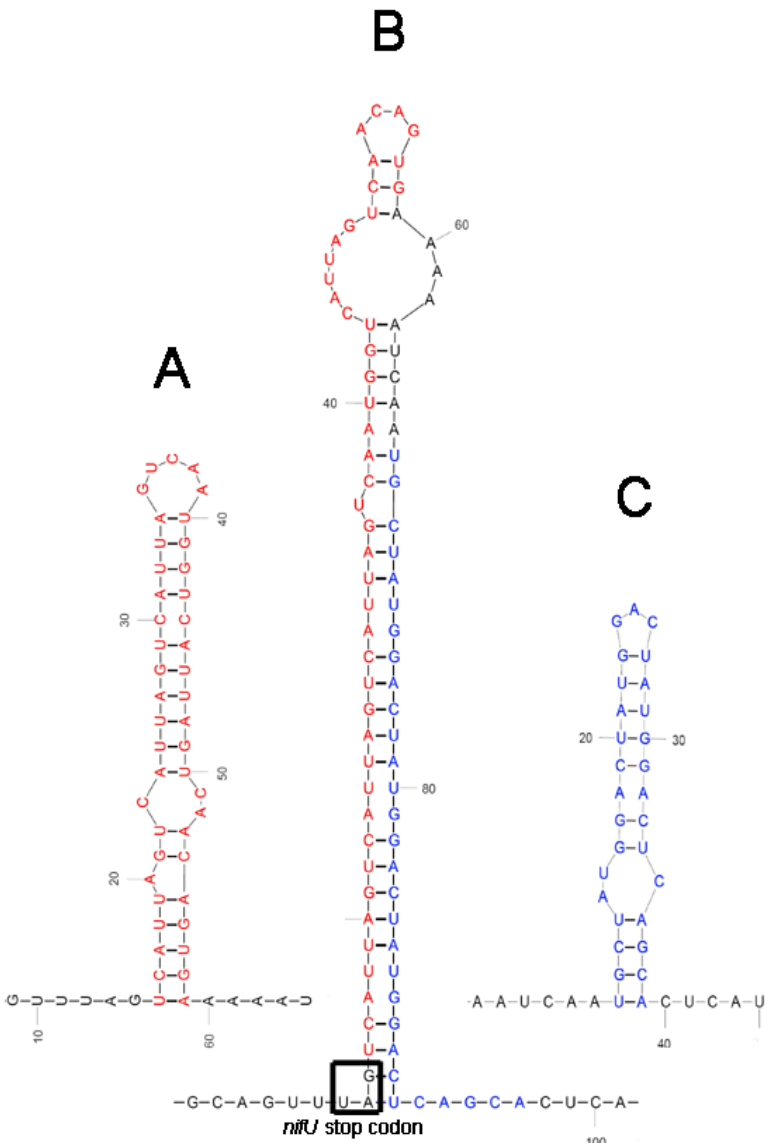



Figure 3.11 Potential secondary structure that can form between the end of *nifU1* and the *nifH1* processing site. (A) The 5' stem loop is shown in red. (C) The 3' stem-loop is shown in blue. (B) A larger structure that can also form from the entire region. Color coded to match Appendix 5.3

## RNA binding proteins

Transcriptional termination/antitermination is often a regulated process (reviewed in Stülke, 2002). Regulation of termination is brought about by alternative stem-loop structures in the 3' UTR of the transcript and often requires RNA binding proteins that assist to stabilize one specific stem-loop relative to the other. Another function of RNA binding proteins is to protect an RNA from degradation, which often requires a stem-loop structure at the 5' end of the transcript to which the RNA binding protein binds. *nifH1* has the potiential to be regulated by one of both of these mechanisms, thus we will study the interaction on RNA binding proteins with the *nifU1-H1* intergenic region further.

Heterocyst forming cyanobacteria have numerous RNA binding proteins compared to only a few that exist in nonheterocystous and unicellular strains (Mulligan *et al.*, 1994). Sato and Wanda demonstrated that a cold regulated RNA binding protein, RpbA1 plays a role in heterocyst differentiation, as a mutation of this gene formed proheterocysts in the presence of nitrate (Sato and Wada., 1996). Furthermore, Mori et al. demonstrated that three RNA binding proteins, *rpbB, rpbC,* and *rpbD,* are regulated by nitrogen status in *Anabaena* (Mori *et al.*, 2003). Transcriptional regulation of nitrogen assimilation by RNA binding proteins has been reported in diazatroph such as *Klebsiella* (Chai and Stewart, 1998) and *Heliobacter* thus it of interest to investigate whether RNA binding proteins may also regulate nitrogen assimilation in *Anabaena*. In fact, in *Heliobacter* a RNA binding protein regulates termination of an *orf1-nifHDK* transcript after *orf1*. This process is N regulated; allowing transcription of *nifHDK* only in the absence of a nitrogen source (Enkh-Amgalan *et al.*, 2006).

We will determine if *rpbA, rpbB, rpbC,* and *rpbD* play a role in nitrogen assimilation or regulation of the nitrogenase by mutating each of these genes in *A. variabilis*. The general effect of these mutations on nitrogen assimilation will be examined by comparing the growth of the mutants on various nitrogen sources ($NH_4$, $NO_3$, $NO_2$, and $N_2$) to that of wild-type. If any of these RNA binding proteins are involved in nitrogen assimilation we expect to observe decreased growth on one or all of alternative nitrogen sources. We will also examine expression of *nifH1* in each of the mutants growing on $NH_4$, $NO_3$, or $N_2$ using qRT-PCR. If one of these RNA binding proteins regulates termination of *nifBSU1*, processing of *nifHDK1,* or stabilization of the *nifHDK1* segment of the transcript we will expect to see a difference in expression of *nifH1* between wild type and the mutant.

The RNA-binding proteins could potentially stimulate or inhibit termination of the *nifBSU1* transcript; and the effect could differ depending on the nitrogen source used. If an RNA binding protein functions to inhibit termination, we will observe decreased expression of *nifHDK1* in the absence of a nitrogen source when that RNA binding protein is mutated. The *nif1B* promoter is expressed at low level in the presence of nitrate (Ehira *et al.*, 2003), however nitrogenase is not expressed in the presence of nitrate; thus if an RNA binding protein functions to stimulate termination, we may observe expression of *nifHDK1* in the presence of nitrate. We do not expect to observe expression of *nifH1* in the presence of ammonia because the *nifB* promoter is not active under this growth condition.

The RNA binding proteins may also affect processing or stability of the *nifHDK1* segment of the transcript. To examine the effect of the RNA binding proteins on stability

of the *nifHDK* transcript, we will examine decay of the *nifH* transcript after transcriptional shutoff using rifampicin in the RNA- binding protein mutants. If we observe altered *nifH1* decay in an RNA binding protein mutant we will verify this effect in-vitro using purified RNA binding proteins (discussed below).

If we identify an RNA binding protein mutant that is impaired in *nifH1* expression we will overexpress that protein in *E. coli* for further study. The RNA binding protein will then be purified and RNA mobility shift will be performed to demonstrate a direct interaction between the RNA binding protein and its hypothetical target mRNA. There are two possible targets for the RNA binding proteins; the stem-loop structures upstream of the *nifH1* transcriptional start site and the stem-loop structure at the 5' end of the *nifH1* transcript. If we observe decreased stability of the *nifH1* transcript in an RNA binding protein mutant then it is likely that this protein functions to stabilize the *nifH1* transcript by binding the stem-loop structure at its 5' end. We will employ an RNA mobility shift using the purified RNA binding protein and an RNA oligo containing the sequences from the *nifH1* 5'UTR that can form the stem-loop structure to demonstrate that the RNA binding protein recognizes this structure on the 5' UTR of *nifH1*. We will then repeat the mRNA decay experiment on *nifH1* with or without the purified RNA binding protein included in the reaction. This will allow us verify that the RNA binding protein stabilizes the mRNA, as the decay rate should be reduced in the presence of the RNA binding protein.

Alternatively, an RNA binding protein may affect nitrogenase activity but not affect *nifH1* decay rates. In this case it is likely that the protein functions in termination/antitermination of *nifU*. To test this hypothesis we will perform RNA

mobility shifts using the purified RNA binding protein and an RNA oligo corresponding to the region that can form the terminator/antiterminator stem-loops. We will also examine RNA oligos from the terminator/antiterminator stem-loop mutants with disrupted base pairing discussed above. This study will allow us to verify that the RNA binding protein interacts with this region and to determine which structure the RNA binding protein interacts with specifically. Together, the data on which stem-loop is bound by an RNA binding protein, the phynotype of the RNA binding protein mutant, and the phenotype of the stem-loop mutant will be used to produce a model for regulation of termination by that RNA binding protien.

### *nifB* promoter

The *nifH1* promoter has long been the focus of studies on transcriptional regulation of *nif* genes. The studies reported here demonstrate that this promoter does not exist and that the *nifB1* promoter is the major promoter driving expression of the *nif* operon. Thus, we will turn our attention to studies on the *nifB1* promoter, which has been poorly characterized. *nifB1* transcripts from *A. variabilis* and its close relatives show a high degree of conservation that extends more than 1 kb upstream from *nifB1*, however the transcriptional start site for *nifB1* is only 350 bp upstream from *nifB1*. Our first study will attempt no narrow down or define the minimal *nifB1* promoter. We will construct various sized *pnifB:lacZ* fusions and examine expression from these constructs in *A. variabilis*. The promoter fragments will be made in 150 bp increments thus we will be able to define the minimal functional *nifB1* promoter to a region of a specific size. The smallest promoter fragment (*frt* cross) that yields expression comparable to wild-type (promoter cross) will be considered the minimal functional *nif* promoter.

Suzuki et al. demonstrated that *nifB1* is regulated by AncrpA, however this regulator does not activate *nifB1* under normal diazatrophic growth. In *Anabaena* many promoters that are differentially regulated based on nitrogen status utilize different transcriptional start sites based on growth conditions. *nifB1* may also utilize a similar mode of regulation, which will be examined further. 5' RACE will be used to map the transcriptional start site of *nifB* in cells growing on different nitrogen sources. We expect that *nifB1* will utilize different transcription start sites depending on growth conditions, an issue that this experiment will resolve.

## Negative regulation of the nitrogenase

Thus far, we have focused on sequence elements that are important for expression of nitrogenase, while ignoring an important aspect of expression, nitrogenase shut-off. Upon the addition of $NH_4$ to an actively fixing culture, nitrogenase activity decreases rapidly even though heterocysts persist for a much longer time. This is caused by inactivation of the nitrogenase protein in conjunction with shut off of transcription of the nitrogenase genes. It is possible that the stem-loops, conserved sequences and/or RNA binding proteins exert their effect during shut-off of the nitrogenase. We will return to the chromosomal mutants and RNA binding protein mutants to examine shut-off of expression of *nifH1* in these mutants. We will use qRT-PCR to examine expression of *nifH1* after the addition of $NH_4$ to actively fixing cultures in the chromosomal mutations that did not previously display a phenotype (Figure 3.2 and stem-loop mutants proposed above). If a sequence element or RNA binding protein is involved in transcriptional shut-of of the nitrogenase, we will observe persistent expression of *nifH1* in that mutant after the addition of $NH_4$.

Another protein that may be involved in negative regulation of *nifH1* is FurA. We have demonstrated that the FurA binding site is not essential for expression of *nifH1* (Figure 3.2), however we have not examined the role of this binding site under conditions of iron limitation. We hypothesize that FurA may attenuate expression of *nifHDK1* when iron is limiting; possibly by affecting formation of the stem-loop structures at the 5' end of the *nifU1-H1* intergenic region. We will create a mutation specifically in the FurA binding site in *A. variabilis.* We will then use qRT-PCR to determine if expression of *nifH1* is altered under conditions of iron limitation in the FurA binding site mutant. If FurA functions to prevent expression of *nifHDK1* when iron is limiting we will expect to see increased expression of *nifH1* in the FurA binding site mutant under conditions of iron limitation.

# SECTION 4 : Regulation of Fructose Transport and Its Effect on Fructose Toxicity in Anabaena spp.

## Introduction

Although the majority of cyanobacteria are obligate photoautotrophs, dependent on sunlight for ATP and on $CO_2$ for carbon, a few well-studied strains can take up and use sugars either only in the light, growing mixotrophically, or in the dark, growing heterotrophically (Smith, 1983). *Synechocystis* sp. strain PCC 6803 is one of the best-studied strains that can grow in the dark using glucose as the sole carbon source; however, it requires short, regular exposure to light for heterotrophic growth (Anderson and McIntosh, 1991). Two well-studied filamentous heterocyst-forming cyanobacterial strains, namely, *Nostoc punctiforme* ATCC 29133 and *A. variabilis* ATCC 29413, are capable of true heterotrophic growth in complete darkness (Haury and Spiller, 1981; Summers *et al.*, 1995). The former strain grows on glucose or fructose, while *A. variabilis* ATCC 29413 can use only fructose (Haury and Spiller, 1981). In these two heterocyst-forming strains, sugars support not only growth in the dark but also nitrogen fixation, an energetically expensive reaction. *N. punctiforme* ATCC 29133 is a symbiont of the bryophyte *Anthoceros punctatus*, and hence, it is likely that its ability to use sugars is essential for its role in symbiosis (Wong and Meeks, 2002). *A. variabilis* is not known to be an endosymbiont; however, by morphology, phenotype, and genetics, it is virtually identical to many strains called *Anabaena azollae*, isolated from the symbiotic

association of cyanobacteria with the water fern *Azolla* (Braun-Howland *et al.*, 1988; Franche and Cohen-Bazire, 1987; Meeks *et al.*, 1988; Zimmerman *et al.*, 1989). Although it was first believed that *A. azollae* was the symbiont, it was shown later that the true symbiont is different and is probably nonculturable (Gebhardt and Nierzwicki-Bauer, 1991). Hence, there is some possibility that *A. variabilis* also once came from *Azolla* and that fructose utilization is associated with symbiosis in this strain. Fructose dramatically affects the physiology of *A. variabilis*. The cells grow faster, are bigger, and in filaments that have differentiated heterocysts, produce more and larger heterocysts, fixing more nitrogen and producing more hydrogen than do cells grown photoautotrophically (Haury and Spiller, 1981; Reddy *et al.*, 1996; Spiller *et al.*, 1983). [$^{14}$C] fructose, which is taken up almost immediately by vegetative cells in a filament, is quickly transported in some form to the heterocysts, where the [$^{14}$C] compound accumulates and is metabolized to provide the reductant for nitrogen fixation (Haury and Spiller, 1981). Although fructose supports nitrogen fixation in whole filaments, isolated heterocysts cannot use fructose as a source of reductant, suggesting either that fructose cannot be transported by heterocysts or that fructose is converted to another compound in the vegetative cell before it moves to the heterocyst (Jensen, 1990). For *N. punctiforme*, a mutant deficient in glucose-6-phosphate dehydrogenase cannot fix nitrogen and cannot grow in the dark with fructose, indicating that the oxidative pentose phosphate pathway is the major pathway for fructose metabolism and is important in heterocysts for nitrogen fixation (Summers *et al.*, 1995). In fructose grown filaments, the heterocysts not only are bigger than those in cells grown photoautotrophically but also store more glycogen and are morphologically different (Lang *et al.*, 1987). Growth with fructose results in increased respiration and decreased

chlorophyll (Pils *et al.*, 2004; Rozen *et al.*, 1986; Valiente *et al.*, 1992). In long-term, dark-grown, fructose-adapted cells, there is an increase in photosystem II, resulting in a decrease in the ratio of photosystem I to photosystem II (Mannan and Pakrasi, 1993). Cells grown with low $CO_2$ in the presence of fructose do not fix $CO_2$ well because of decreased carbonic anhydrase and decreased ribulose bis-phosphate carboxylase oxygenase (Nieva and Valiente, 1996). The decrease in oxygen production in fructose-grown cells is thought to contribute to a micro-oxic environment that better supports nitrogen fixation (Haury and Spiller, 1981). Microarray analysis of RNA from the non-nitrogen-fixing unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 under conditions of nitrogen starvation shows increased expression of genes important in glycolysis, the oxidative pentose phosphate pathway, and glycogen catabolism and increased activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, two key enzymes of the oxidative pentose phosphate pathway (Osanai *et al.*, 2006). In *Synechocystis*, transcription of the genes for sugar catabolism is regulated by Hik8 (Singh and Sherman, 2005), a homolog of a protein (SasA) in *Synechococcus* that regulates *kaiC*, which is part of the central oscillator of circadian rhythm (Iwasaki *et al.*, 2000; Kondo, 1994, 1993). In addition, the sigma factor SigE positively regulates three glycolytic genes, four oxidative pentose phosphate genes, and two glycogen metabolism genes (Osanai *et al.*, 2005). Activation of sugar catabolic genes under conditions of nitrogen starvation requires the global nitrogen activator NtcA (Osanai *et al.*, 2007; Osanai *et al.*, 2006). SigE in *Anabaena* sp. strain PCC 7120, a strain that cannot use fructose, is not essential for nitrogen fixation but is expressed late in heterocyst differentiation, suggesting that it has a role in heterocyst function (Aldea *et al.*,

2007; Khudyakov and Golden, 2001). The transport of glucose in *Synechocystis* is known to occur via a glucose-fructose permease, the product of the *glcP* transport gene (Flores and Schmetterer, 1986; Joset *et al.*, 1988; Zhang *et al.*, 1989). Transport of fructose is toxic to the cells; inactivation of *glcP* relieves the toxicity but no longer allows the cells to grow using glucose (Flores and Schmetterer, 1986; Joset *et al.*, 1988). Expression of *glcP* in *Synechococcus* sp. strain PCC 7942 resulted in a strain that was capable of glucose transport but also died in the presence of glucose (Zhang *et al.*, 1998). Uptake of fructose in *A. variabilis* and *N. punctiforme* is constitutive but increases after exposure to fructose (Schmetterer and Flores, 1988) and is energy dependent in *A. variabilis* (Valiente *et al.*, 1992). The $K_m$ for fructose uptake is about 160 μM for cells that have not been grown with fructose and about 50 μM for cells pregrown with fructose, and it does not change in the light versus the dark (Jensen, 1990). Described here are the genes for fructose transport in *A. variabilis*, their regulation, and the effect of their expression on growth of the obligately photoautotrophic strain *Anabaena* sp. strain PCC 7120.

# Results

## Identification of fructose transport genes.

*A. variabilis, N. punctiforme* and *Anabaena* sp. PCC 7120 are similar species of heterocyst forming cyanobacteria. *A. variabilis and Anabaena* sp. 7120 share 95-100% amino acid identity within their coding regions while *A. variabilis* and *N. punctiforme* share 90-95% amino acid identity (IMG). Both *A. variabilis* and *N. punctiforme* can utilize fructose as a carbon source when growing heterotrophically in the dark, while *Anabaena* sp. PCC 7120 cannot. The *A. variabilis* genome consists of approximately 5750 genes; ~4500 of these genes are present in all three species, ~5100 of the 5750 genes are shared with only *Anabaena* sp. PCC 7120, while only 4750 of the genes are shared only *N. punctiforme* (IMG). The fructose transport genes were identified using a bio-informatics approach to compare the genomes of these three species. Fructose transport genes were identified as putative ABC-type sugar transport genes present in the genomes of *A. variabilis* and *N. punctiforme*, both capable of heterotrophic growth in the dark with fructose as the sole carbon source, but not in the genome of *Anabaena* sp. strain PCC 7120, an obligate photoautotroph (Figure 4.1). The organization of the ABC-type transport genes, *frtABC*, and a *lacI*-like regulatory gene, *frtR*, is shown in Fig. 4.2. *ava2171*, here-in named *frtA*, is the putative periplasmic component of an ABC sugar transporter. *ava2172*, here-in named *frtB*, is the putative ATPase component of an ABC transporter. *ava2173*, here-in named *frtC*, is the putative inner membrane translocator of an ABC transporter. Immediately upstream of these genes and transcribed in the opposite direction is a putative *lacI*-like transcriptional regulator, here-in named *frtR*. The gene flanking *frtR* is tentatively identified as encoding dihydrouridine synthase and has

close homologs in the genomes of *Anabaena* sp. PCC 7120, *N. punctiforme*, and *Nodularia spumigena* CCY9414, (another heterocyst-forming strain that cannot use sugars for growth) while the *ava2174* gene encodes a putative porin-like protein. There is a close homolog of the *ava2174* gene in the genome of *N. punctiforme* but not in the genomes of *Anabaena* sp. PCC 7120 and *N. spumigena*. The homolog of FrtR in *N. punctiforme* (73% identical) is HrmR (Npun02008536), a repressor of its own gene, *hrmR*, and of *hrmE* (Npun02008530), which are genes involved in the differentiation of hormogonia in *N. punctiforme* (Campbell, 2003). There is no homolog of *hrmE* in *A. variabilis* or in the genomes of any other sequenced cyanobacteria. The homologs of FrtABC in *N. punctiforme* (Npun02008528, Npun02008527, and Npun02008526 to Npun02006538), with 72 to 85% identities to the proteins in *A. variabilis*, are downstream of *hrmE* in the *N. punctiforme* genome. Downstream of the *frtABC* homologs in *N. punctiforme* is a gene (Npun02006539) that appears to encode a glucose transporter. This gene is absent from *A. variabilis*, consistent with the fact that this strain cannot use glucose as a carbon source; however, there is a homolog (sll0771; named *glcP*

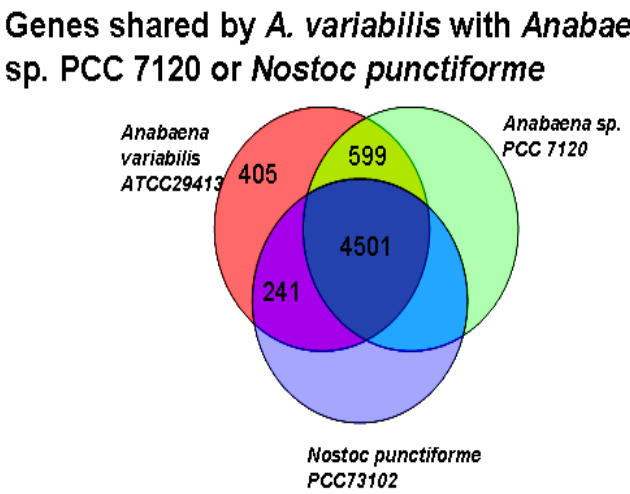



**Figure 4.1** Genome comparison of three heterocyst forming cyanobacteria.

for glucose permease) with 71% identity in *Synechocystis* sp. strain PCC 6803, a strain that can grow heterotrophically in the dark with glucose. The *hrmE* gene, which is involved in regulating hormogonia differentiation in *N. punctiforme,* is regulated by HrmR, a homolog of FrtR (Campbell *et al.*, 2003). Although *hrmE* and *frtA* are not similar, nor do they share similar functions, their regulatory regions show a high degree of similarity (Figure 4.2). The region of *hrmE* that contains the transcription start site and the two binding sites for HrmR is well conserved with the homologous the region upstream of *frtA* in *A. variabilis* (Figure 4.2), and the two binding sites for HrmR are nearly identical between *frtR* and *hrmE*. In the regulatory region of *frtA*, the second

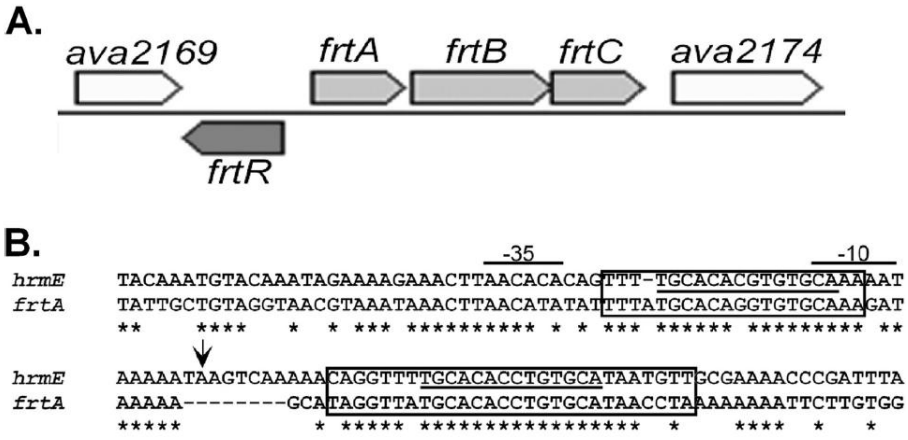


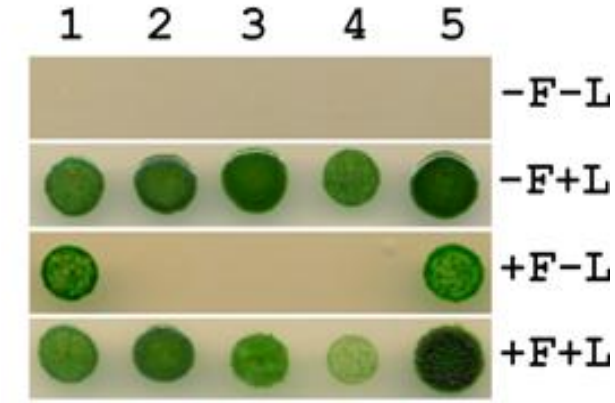

**Figure. 4.2** Fructose transport genes. (A) The region of the chromosome of *A. variabilis* with the fructose transport genes, namely, *frtR* (*ava2170*), encoding a putative *lacI*-like transcriptional regulator, and *frtABC* (*ava2171* to *-2173*), encoding a putative periplasmic binding component, ATPase component, and transmembrane component, respectively. (B) Alignment of the promoter region of *hrmE* of *N. punctiforme* with a conserved region of the *frtA* promoter region, beginning about 300 bp upstream from the start codon of *frtA*. The transcription start site of *hrmE* is indicated by an arrow, and the -10 and -35 regions of the *hrmE* promoter are labeled. The HrmR binding sites, which are underlined, are shown within boxes that indicate longer conserved palindromic sequences.

HrmR-like binding site is within a larger, 28-bp, almost perfect palindrome (Figure 4.2); however, that larger palindrome is not as well conserved in *hrmE*. In contrast, in *hrmE*, the first HrmR-binding site is within a 20-bp perfect palindrome (Figure 4.2) that is not as well conserved in *frtA*. The striking similarity in these regions suggests that *hrmE* and *frtA* have similar modes of regulation, which is supported by the similarity of FrtR and HrmR proteins. There are two HrmR-binding sites in the promoter region of *hrmR*, and HrmR binds to this region; thus, it is autoregulatory (Campbell *et al.*, 2003). Since *frtA* and *frtR* in *A. variabilis* are divergent genes (Figure 4.2), they share a regulatory region and therefore share the two HrmR binding sites shown in (Figure 4.2).

## Function of *frtRABC*.

*frtRABC* are annotated as "putative sugar transport system," however annotations are often incorrect, thus their actual function was verified experimentally. In order to determine whether the *frtRABC* genes function to transport fructose, *frtRABC* or *frtABC*, lacking *frtR*, was transferred to *Anabaena* sp. PCC 7120, a strain that lacks the *frtRABC* genes and cannot grow heterotrophically in the dark with sugars. An *frtR* mutant of *A. variabilis* was also constructed. The wild-type strain of *A. variabilis* grew well in the light with or without fructose but grew in the dark only in the presence of fructose (Figure 4.3, lane 5). In contrast, *Anabaena* sp. PCC 7120 grew only in the light and could not grow in the dark with fructose (Figure 4.3, lane 4) unless the strain also contained the *ftrRABC* genes of *A. variabilis* (Figure 4.3, lane 1). Thus, the *frtRABC* genes in *Anabaena* sp. PCC 7120 were sufficient to allow the strain to transport fructose. The great genetic similarity of these two species suggests that they share the same metabolic pathways;

thus, in *Anabaena* sp. PCC 7120, the only barrier to the utilization of fructose in the dark is the inability of the strain to transport the sugar. The *frtR* gene was essential for growth in the dark with fructose; neither the *frtR* mutant of *A. variabilis* (Figure 4.3, lane 3) nor a mutant of *Anabaena* sp. PCC 7120 containing only *frtABC*, without *frtR*, was able to grow in the dark in the presence of fructose. These results suggested that FrtR was essential for expression of *frtABC* and might be an activator; however, this was not consistent with its similarity to the LacI repressor and to HrmR, which is also a repressor, so I explored this further.




Data kindly provided by Brenda Pratte

**Figure 4.3**. Growth of strains with or without fructose transport genes.Cells of *A. variabilis* strain FD or *Anabaena* sp. strain PCC 7120 with or without *ftrRABC* genes were grown on BG-11 agar medium with or without 5 mM fructose for 4 days in the light or 7 days in the dark. Lane 1, *Anabaena* sp. strain PCC 7120 BP291, containing the *frtRABC* genes; lane 2, *Anabaena* sp. strain PCC 7120 BP292, containing the *frtABC* genes; lane 3, *A. variabilis* BP301 (*frtR* mutant); lane 4, *Anabaena* sp. strain PCC 7120; lane 5, *A. variabilis* strain FD.

## Transcription of *frtA* and *frtR*.

If the *frtRABC* genes encode a fructose transport system, then they would likely be regulated by fructose. RT-PCR was used to examine transcription of the *frtR* and *frtA* genes of *A. variabilis* as well as *Anabaena* sp. PCC 7120 with *frtRABC* or just *frtABC* in response to fructose. The expression of *frtR* and *frtA* in response to fructose was also

examined in the *frtR* mutant and the *frtR* overexpression strains of *A. variabilis*. Analysis of transcripts of *frtA* and *frtR* in cells grown with or without fructose indicated that in both *A. variabilis* and *Anabaena* sp. PCC 7120 with *frtRABC*, transcription of *frtA* and *frtR* was induced by fructose, although low levels of transcript were detected for both genes in the absence of fructose. *frtA* was more strongly induced by fructose than *frtR* (see Figure 4.4). If FrtR were an activator, then transcription of *frtABC* would require FrtR. However, FrtR is a repressor, since in both the *frtR* mutant of *A. variabilis* and in *Anabaena* sp. PCC 7120 with *frtABC* but not *frtR*, expression of *frtA* is constitutive (Figure 4.4, lanes 5 to 8). Further supporting the role of FrtR as a repressor was the

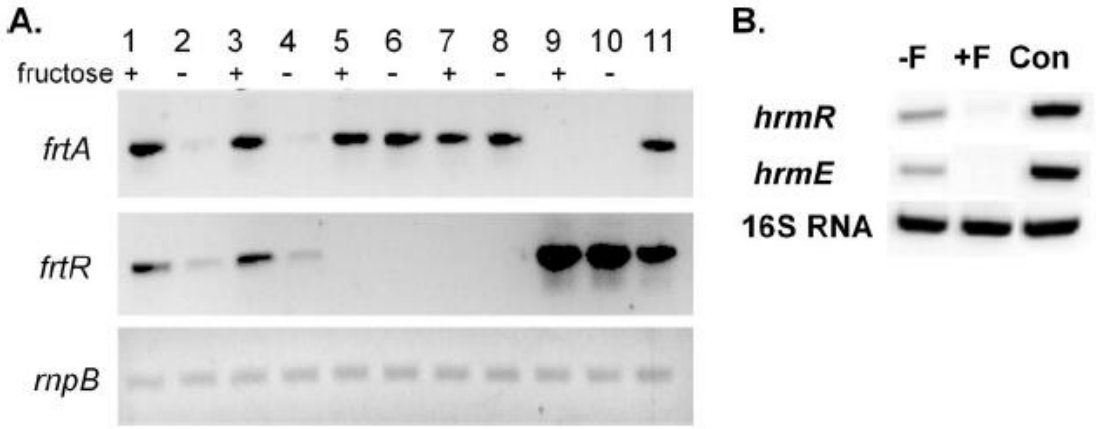



**Figure 4.4** Transcription of *frtA, frtR, hrmR,* and *hrmE*. (A) Transcription of *frtA* and *frtR* was determined by RT-PCR, using RNAs extracted from *A. variabilis* strains grown with or without 5 mM fructose for 24 h. Lanes 1 and 2, wild-type *A. variabilis*; lanes 3 and 4, BP291 (*Anabaena* sp. Strain PCC 7120 with *frtRABC*); lanes 5 and 6, BP292 (*Anabaena* sp. strain PCC 7120 with only *frtABC*); lanes 7 and 8, BP301 (*A. variabilis frtR* mutant); lanes 9 and 10, BP356 (*A. variabilis* strain overexpressing *frtR*); lane 11, positive control (FD DNA). Transcription of *rnpB* was the control for equal amounts of RNA in each reaction. (B) Transcription of *hrmR* and *hrmE* was determined by RT-PCR, using RNAs extracted from *N. punctiforme* grown in AA/8 without (F) or with (F) 5 mM fructose for 24 h. Con, positive control using chromosomal DNA from *N. punctiforme.* 16S rRNA was the control for equal amounts of RNA in each reaction.

finding that in a strain of *A. variabilis* that overexpressed *frtR*, there was no expression of *frtA*, with or without fructose (Figure 4.4, lanes 9 and 10).

## Regulation of *hrmR* by fructose.

Since the homologue of *frtR* in *N. punctiforme* is *hrmR*, which has been shown to regulate itself and another gene, *hrmE* (Campbell *et al.*, 2003), I used RT-PCR to determine whether these genes were also induced by growth with fructose. Both genes were repressed by fructose (Figure 4.4). Since the results were the reverse of those expected, the entire experiment was done a second time, beginning with new cultures of *N. punctiforme* grown with or without fructose, but the results were the same. Thus, despite the similarity in the encoded proteins, *frtR* and *hrmE* respond differently to fructose. This may be a result of the different functions of HrmR and FrtR. In *N. punctiforme* HrmR does not regulate the fructose transport genes and its expression is poorly modulated by fructose.

## Expression of *frtA-lacZ* and *frtR-lacZ* fusions.

The *frtR* and *frtABC* genes are located ~400bp apart and are divergently transcribed. This means that both promoters are in the same intergenic region. Additionally, the binding sites for *frtR* are a palandromic; thus' their sequence is the same in both promoters and these sites regulate both divergent promoters simultaneously and coordinately. The *frtA* and *frtR* promoters were fused to *lacZ* to quantitate changes in expression of these genes in response to fructose in the presence and absence of FrtR. The effect of FrtR on its own promoter was measured by examining a *pfrtR:lacZ* fusion

in a *frtR* mutant and wild-type. Two fusions were created for each gene. In the first type, a promoterless *lacZ* gene was inserted into *frtA* and *frtR*, thus providing not only a normal promoter but also a normal context for the promoter in the chromosome. The second type of fusion placed a 400-bp fragment consisting of the *frtR-frtA* intergenic region in front of *lacZ* in both orientations, and then the entire construct was integrated into a neutral site on the chromosome. This effectively created both *pfrtA* and *pfrtR* :*lacZ* fusions. For the first type of fusion, expression of *frtA* (strain BP352), as measured by ß-galactosidase activity, increased about 30-fold with fructose, while expression of *frtR* (strain BP353) increased about 7-fold with fructose (Figure 4.5). For the second type of fusion, expression of *frtA* (strain JU338) increased about four-fold with fructose, while expression of *frtR* (strain JU336) increased about five-fold (Figure 4.5). Even though the promoter fragments used were large and should have had all the necessary *cis*-acting elements, expression of *frtA* and *frtR* in the BP352 and BP353 strains, in which the fusions were in the normal chromosomal locations, was more highly expressed than that in the counterpart strains JU338 and JU336, which had the promoter-*lacZ* fusion integrated at a different site by single-crossover recombination. This may be due to differences in gene expression from the different chromosomal locations or our inability to fully segregate the *pfrtA:lacZ* fusions onto every copy of the chromosome. Consistent with the results from RT-PCR (Figure 4.5), in the presence of fructose the expression of *frtA* was higher than the expression of *frtR*, and the expression of *frtA* was more strongly induced by fructose than the expression of *frtR*.

To determine the effect of FrtR on expression of *frtA* and *frtR*, the promoter fragment fusions were also constructed in an *frtR* mutant of *A. variabilis* (BP301) and in

*Anabaena* sp. PCC 7120, which naturally lacks *frtR*. In the absence of FrtR, *frtA* expression, as measured by ß-galactosidase activity, was about 10-fold higher than in the wild-type strain (compare strains JU355 and JU338) (note the difference in scale of the *y* axes in Figure 4.5) and was unaffected by the presence of fructose (Figure 4.5). In the absence of FrtR, *frtR* expression, as measured by ß -galactosidase activity, was about 25-fold higher than in strains with FrtR (compare strains JU353 and JU336) and was also unaffected by the presence of fructose (Figure 4.5). This indicates that in the presence of fructose, FrtR represses itself more strongly than it represses *frtA*. For both *frtA* and *frtR*, constitutive expression in the absence of FrtR was about 1.5-fold higher in *Anabaena* sp. PCC 7120 than in the *frtR* mutant of *A. variabilis* (BP301) (Figure 4.5). The difference between expression in the two strains is likely a result of the fact that in *Anabaena* sp. PCC 7120 the constructs are on a multicopy plasmid but since *A. variabilis* does not maintain plasmids, the same fusions were incorporated into the chromosome in these strains. The constitutive expression of *frtA* and *frtR* in the absence of FrtR provides further evidence that FrtR is a repressor. In strains with FrtR, expression of *frtA* and *frtR* was much more strongly repressed, even in the presence of fructose, than that in strains lacking FrtR, indicating that there is some level of repression of *frtA* and *frtR* by FrtR under all growth conditions tested. In the strains with FrtR in which *frtA* and *frtR* were expressed with fructose, *frtA* was more strongly expressed than *frtR*. However, in the absence of FrtR, the difference in expression between *frtA* and *frtR* was much smaller, suggesting that the lower level of *frtR* expression in the strains with FrtR was the result of stronger repression of *frtR* than of *frtA* in the presence of fructose and not the result of a much stronger promoter for *frtA*. Together, these results indicated that FrtR repressed

expression of both *frtA* and *frtR* in the presence or absence of fructose, but the repression was much weaker in the presence of fructose.

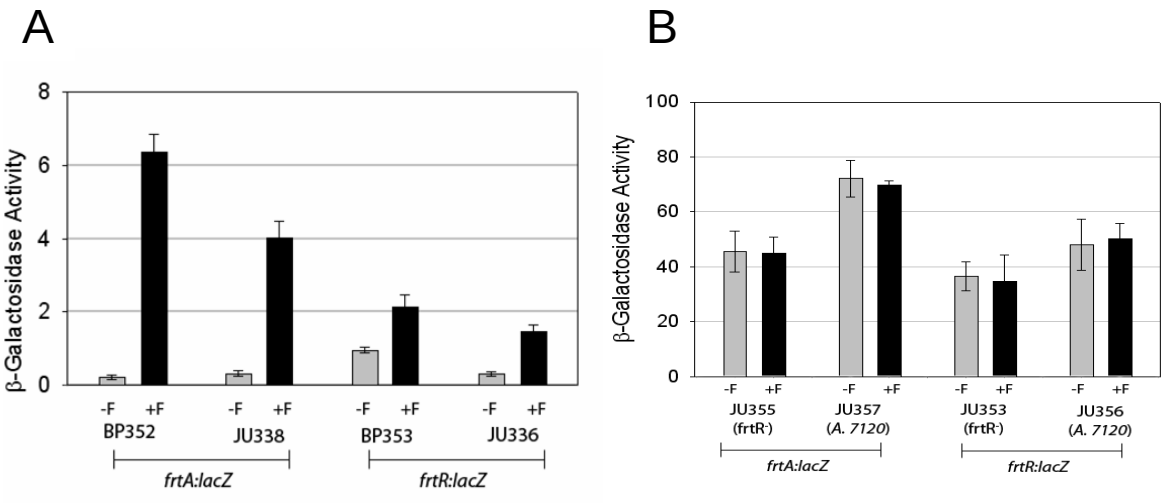



Figure **4. 5** Expression of *frtA-lacZ* and *frtR-lacZ* fusions. (A) Relative rates of β-galactosidase activity were measured in strains BP352 (*lacZ* inserted within the *frtA* gene), JU338 (containing a 500-bp *frtA* promoter fragment fused to *lacZ*), BP353 (*lacZ* inserted within the *frtR* gene), and JU336 (containing a 400-bp *frtR* promoter fragment fused to *lacZ*), grown in AA/8 with or without 5 mM fructose. (B) Relative rates of β-galactosidase activity were measured in strains JU355 (containing a 500-bp *frtA* promoter fragment fused to *lacZ* in strain BP301, the *frtR* mutant), JU357 (containing a 500-bp *frtA* promoter fragment fused to *lacZ* in *Anabaena* sp. strain PCC 7120), JU353 (containing a 400-bp *frtR* promoter fragment fused to *lacZ* in strain BP301, the *frtR* mutant), and JU356 (containing a 400-bp *frtR* promoter fragment fused to *lacZ* in *Anabaena* sp. strain PCC 7120), grown with or without 5

## Binding of FrtR to the *frtR-frtA* promoter region.

Recombinant FrtR was purified from *E. coli* as inclusion bodies, and the protein was renatured. Electrophoretic mobility shift assays were used to verify that FrtR binds the promoters of *frtA* and *frtR*. The protein bound to two sites on a DNA fragment that included the intergenic region between *frtR* and *frtA* (Figure 4.6). This region includes the two HrmR-like binding sites shown in Fig. 4.2. The binding was competed using the

same cold DNA fragment but was not competed using an unrelated DNA fragment from the *rnpB* gene. The addition of fructose to the binding reaction mix had no effect on the mobility shift. This may be due to binding of FrtR to this region even in the presence of fructose. This is evident from the repression of *frtA* and *frtR* by FrtR even in the presence of fructose, as shown by the much higher levels of expression of *frtA-lacZ* and *frtR-lacZ* in an *frtR* mutant than in the wild-type strain in the presence of fructose (Figure 4.6). The fact that fructose did not affect binding of FrtR in the EMSA is in agreement with the fact that FrtR retains most of its repressor activity even in the presence of fructose.

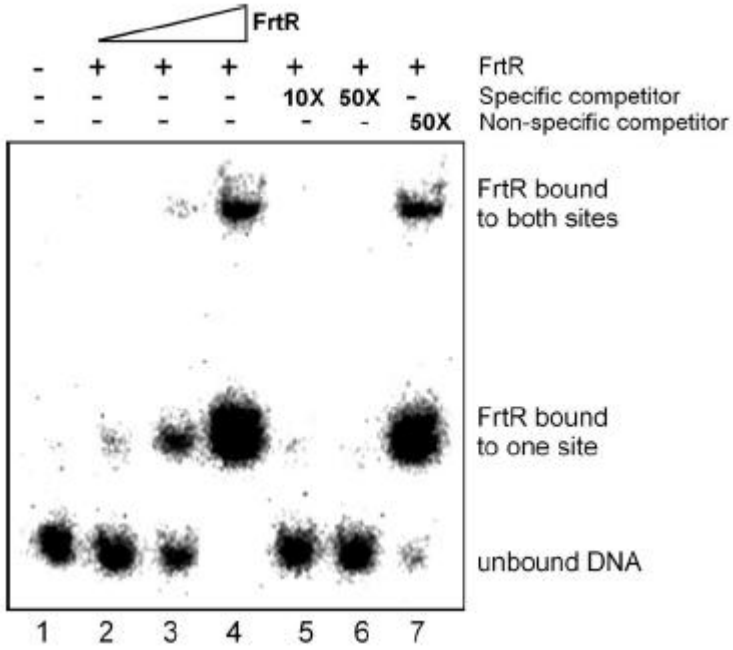



**Figure 4.6** Binding of FrtR to the promoter region of *frtA*. A 32P labeled 131-bp DNA fragment upstream of *frtA* was incubated with or without recombinant FrtR protein. Samples in lanes 2 and 3 contained 100 ng and 300 ng of FrtR protein extract, respectively. Samples in lanes 4 to 7 contained 700 ng of FrtR protein extract.

## Growth of strains with fructose.

As shown in Fig. 4.3, neither the *frtR* mutant of *A. variabilis* nor the mutant of *Anabaena* sp. PCC 7120 with *frtABC* but without *frtR* grew in the dark with fructose, suggesting that FrtR might be an activator. However, the transcription data shown in Fig.

4.4 and 4.5 demonstrate that FrtR is a repressor. *Anabaena* sp. PCC 7120 grew poorly in the light in the presence of fructose but not in its absence (Figure 4.7). When cells are growing in the light with $CO_2$, carbon is not limiting, thus the reduced growth observed in *Anabaena* sp. PCC 7120 is likely a result of sugar toxicity. Generally, sugars are toxic to cyanobacteria with the exception of a few symbiotically competent strains such as *A. variabilis* and *N. punctiforme* (Meeks, 2006). I hypothesize that sugar transport must be limited, even in strains that can grow heterotrophically with fructose, to prevent sugar toxicity resulting from excessive sugar transport. Two lines of evidence support this hypothesis. First, the fructose transport genes remain repressed even under inducing conditions (+ fructose). Second, deletion of the repressor produces a strain that cannot grow in the dark on fructose (Figure 4.3), even though the transport genes are highly expressed (Figure 4.4). To investigate the effects of fructose toxicity on growth I grew the wild-type strain of *A. variabilis* and the *frtR* mutant with various concentrations of fructose in the light. The wild-type strain grew much better with fructose than without it, whereas the *frtR* mutant did not (Figure 4.7). For the wild-type strain, increasing concentrations of fructose, from 1 to 50 mM, supported increased growth rates, but 200 mM fructose decreased the growth rate to about the same rate as that with 1 mM fructose (Figure 4.7). For the *frtR* mutant, increasing concentrations of fructose did not increase the growth rate (Figure 4.7; note the difference in scale of the *y* axis). In fact, concentrations of fructose above 1 mM decreased growth, and 200 mM fructose almost completely inhibited growth. Strain JU377, in which the *frtABC* genes were overexpressed from the strong *psbA* promoter in a wild-type *frtR* background, grew poorly even in the absence of fructose and died after exposure to even 1 mM fructose

(Figure 4.7). This indicates the inability of the *frtR* mutants to grow heterotrophically on fructose is not a result of the loss of FrtR or its ability to regulate an unidentified gene and that it is caused specifically by over expression of *frtABC*. Thus, it appears that overexpression of *frtABC* in the *frtR* mutant leads to transport of fructose at levels that are toxic to the cells. In the light, the *frtR* mutant was apparently able to overcome this toxicity when the concentration of fructose was low, but in the dark, when the cells were dependent on fructose as a carbon source, even 5 mM fructose was toxic (Figure 4.7, lane 3), suggesting that metabolism of fructose was involved in the toxic effect of high intracellular concentrations of fructose. Even without fructose, the poor growth of strain JU377, which overexpressed *frtABC*, suggested that excessive amounts of the transporter itself are also deleterious to cell growth.

*Anabaena* sp. PCC 7120 mutant BP291 (with *frtRABC*) grew in the dark with fructose (Figure 4.7); however, it grew more slowly than *A. variabilis* FD (Fig. 4.7). However, unlike the growth of *A. variabilis* FD in the light, the growth of BP291 in the light was not enhanced by fructose (Figure 4.7), and 50 mM fructose, which greatly stimulated growth of FD (Figure 4.7), slightly inhibited growth of BP291 (Figure 4.7). *Anabaena* sp. PCC 7120 with *frtRABC* grew in the light with 5 mM fructose and the PSII inhibitor DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], but growth was slow, particularly under nitrogen-fixing conditions, and increasing the fructose concentration above 5 mM did not help the growth (data not shown). Thus, although *Anabaena* sp. PCC 7120 mutant BP291 was able to transport and use fructose in the dark, in the light the strain did not use the fructose and grew photoautotrophically. *Anabaena* sp. PCC 7120 mutant BP292 (with *frtABC* but lacking *frtR*) did not grow in the dark with 5 mM

fructose (Figure 4.7, lane 2) and died in the light with as little as 1 mM fructose (Figure 4.7), showing even greater sensitivity to fructose than that of BP301, the *A. variabilis* strain lacking *frtR* (Figure 4.7). Unregulated expression of *frtABC* led to a complete inhibition of growth, suggesting that fructose is toxic to this strain in the light.

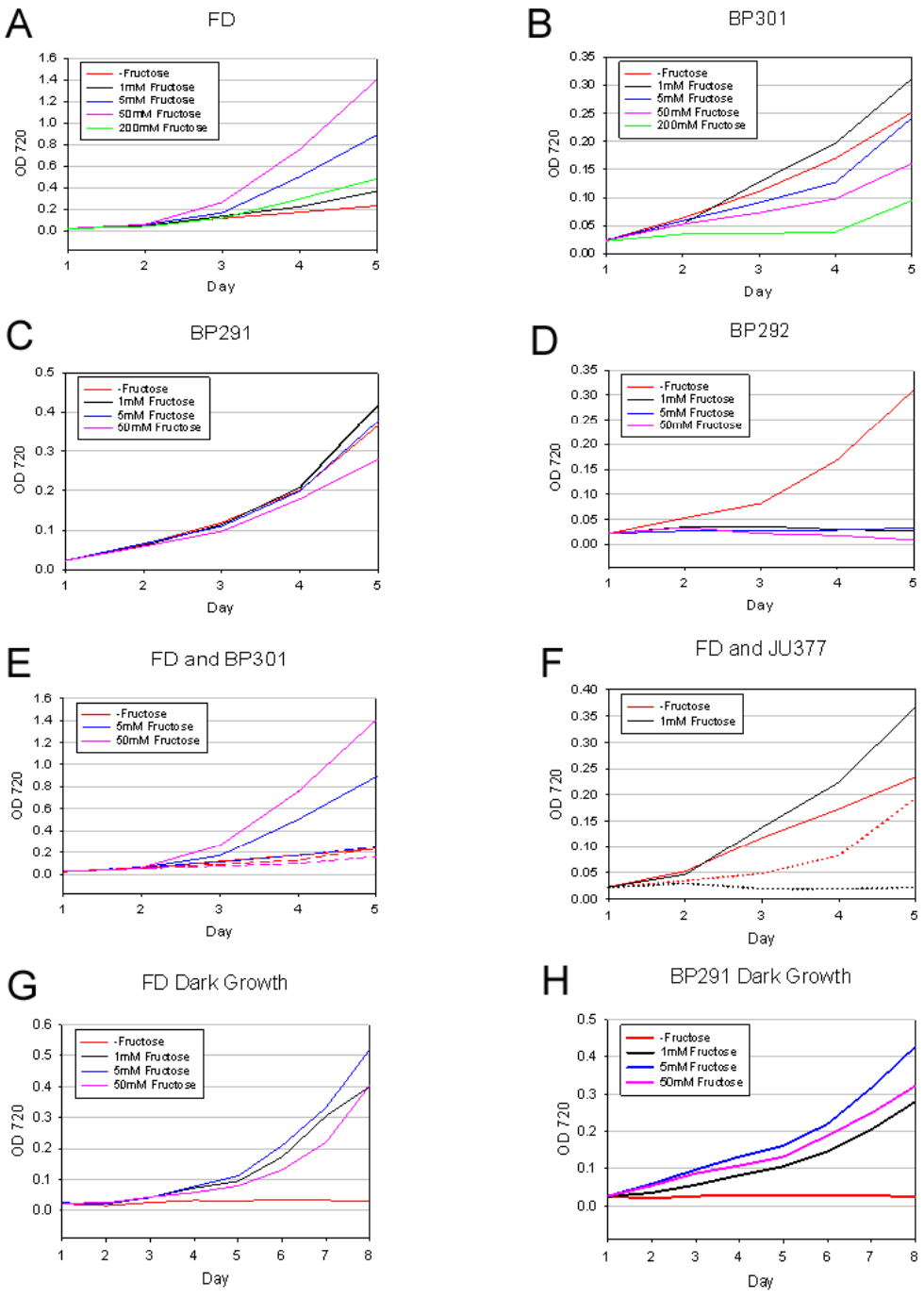



**Figure 4.7** Growth of strains with fructose. The strains indicated for each panel were grown in AA/8 without fructose and then diluted in medium containing the concentrations of fructose indicated by the symbols on day 0. (A) Strain FD (*A. variabilis* wild type (B) ) BP301 (*frtR* mutant). (C) *Anabaena* sp. strain PCC 7120 with the *frtRABC* genes (strain BP291) (D) *Anabaena* sp. strain PCC 7120 with the *frtABC* genes (lacking *frtR*) (strain BP292). (E) FD (solid lines) and BP301 (dashed lines) (F) FD (solid lines) and JU377 (dotted lines), a strain of *A. variabilis* in which the *frtABC* genes are constitutively expressed from the strong *psbA* promoter. (G) Wild-type strain FD grown in the dark. (H) *Anabaena* sp. strain PCC 7120 with the *frtRABC* genes (strain BP291) grown in the dark.

**Uptake of fructose in the *frtR* mutant.**

Overexpression of *frtABC* in BP301 (*frtR* mutant) might be expected to affect the rate of fructose uptake. I measured fructose uptake by the disappearance of fructose from the medium in the wild-type strain and in BP301 (*frtR* mutant). In the first 2 hours after the addition of fructose, the rate of uptake was greater in the BP301 mutant, but the initial high rate slowed for both strains about 2 hours after the addition of fructose (Figure 4.8). BP301 continued to take up fructose slightly faster than the wild-type strain for even up to 8 h. In the absence of the repressor, FrtR, high levels of expression of *frtABC* allowed the uptake of fructose to begin immediately upon its addition, while the wildtype strain showed a lag in uptake of about 40 min (Figure 4.8). This suggests that while *frtABC* is transcribed at low levels even in the absence of fructose, fructose is required for synthesis of sufficient FrtABC to efficiently transport fructose. In the absence of the repressor (strain BP301), sufficient FrtABC is made to allow the immediate transport of fructose.

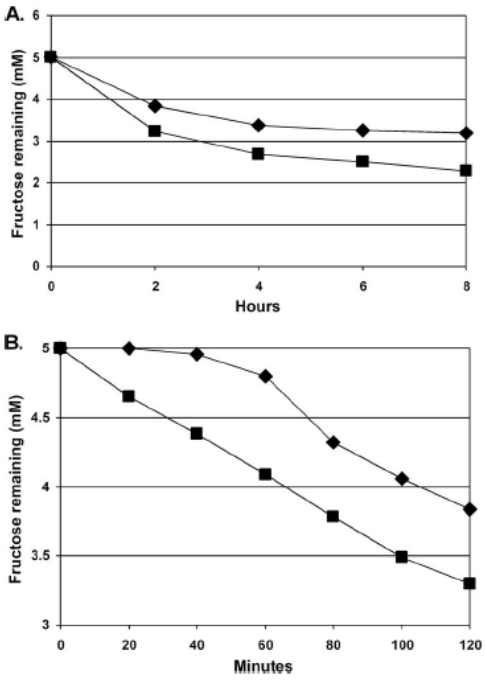



**Figure 4.8** Fructose uptake in wild-type (diamond) and BP301 (*frtR* mutant) (square) strains. Cells were grown in AA/8 to an $OD_{720}$ of 0.250, fructose was added at time zero, and transport was measured as the disappearance of fructose from the medium over time (hours [A] or minutes [B]). Fructose was measured using a fructose assay kit (Sigma-Aldrich).

**Filaments and heterocysts of fructose-grown cells.**

In *A. variabilis* fructose has been reported to increase the number, size, and frequency of heterocysts, increase nitrogen fixation, and cause increased glycogen storage in heterocysts. This is consistent with both the strain's ability to use fructose as a carbon source in the light and its ability to grow symbiotically with a plant partner. (Haury and Spiller, 1981; Lang *et al.*, 1987). I was curious if the closely related strain *Anabaena* sp. PCC 7120, which normally cannot grow with fructose as a carbon source or form symbiotic relationships, exhibited similar phenotypes to *A. variabilis* after gaining the ability to grow on fructose. Forty-eight hours after nitrogen stepdown, *A. variabilis* strain FD grown with 50 mM fructose had 11.9% heterocysts, compared to only 7.6% in filaments grown without fructose. In contrast, under the same growth conditions, BP291, the *Anabaena* sp. PCC 7120 mutant with *frtRABC*, produced 7.1% heterocysts with fructose and 6.8% heterocysts without fructose. Also, in contrast to the increased size of cells in filaments of *A. variabilis* FD grown with 5 mM fructose, filaments of BP291 showed no increase in size with 5 mM fructose (Figure 4.9), and in contrast to *A. variabilis*, the strain showed no increase in the rate of nitrogen fixation when grown with fructose (data not shown). These results suggest that the inability of *Anabaena* sp. PCC 7120 to utilize fructose goes beyond its inability to take up the sugar; and although *Anabaena* sp. PCC 7120 and *A. variabilis* can both use fructose for heterotrophic growth, *A. variabilis* has specific adaptations for heterotrophic growth that do not exist in *Anabaena* sp. PCC 7120.

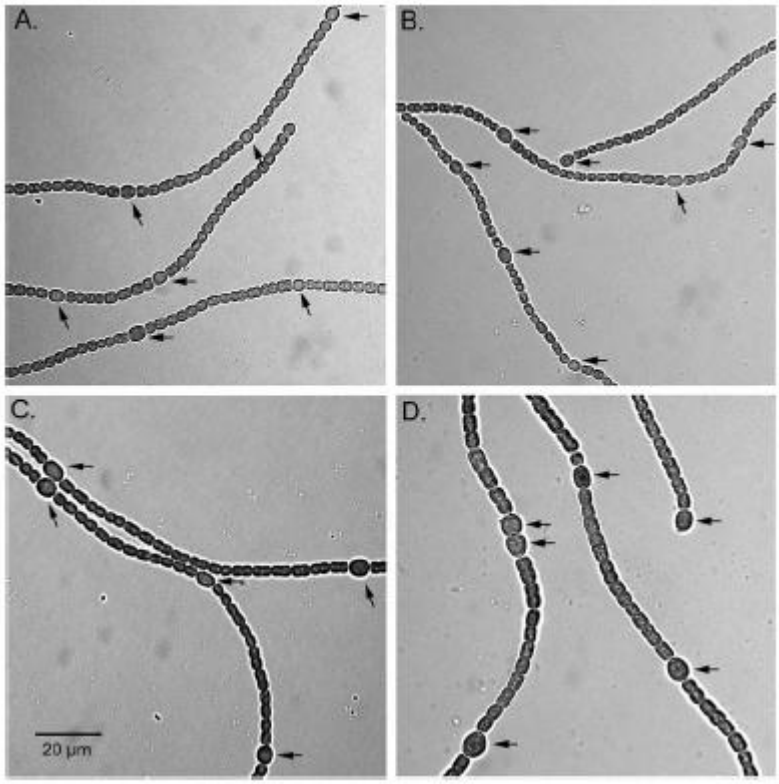



**Figure 4.9** Light micrographs of filaments of *Anabaena* sp. strain PCC 7120 with the *frtABC* genes (strain BP291) grown without (A) or with (B) 5 mM fructose and of filaments of *A. variabilis* FD grown without (C) or with (D) 5 mM fructose. The size scale is the same for all panels. Heterocysts are indicated by arrows.

# Discussion

The normal Frt$^-$ phenotype of *Anabaena* sp. PCC 7120 was complemented by the addition of the *A. variabilis* fructose transport genes, *frtRABC*. Therefore, the lack of a fructose transport system in *Anabaena* sp. PCC 7120 was the only barrier to heterotrophic growth on fructose in the dark. However, both *Anabaena* sp. 7120 BP292, which constitutively expressed *frtABC* due to a lack of the repressor FrtR, and *A. variabilis* strain BP301, a *frtR* mutant, were unable to grow on fructose in the dark even though they expressed *frtABC* and transported fructose. These results indicate that the repressor, FrtR, is required for heterotrophic growth on fructose.

The evidence presented here suggests that the lack of FrtR caused excessive fructose uptake via the high-level constitutive expression of the transport genes and that this led to toxicity. Fructose is toxic in two cyanobacterial strains that have glucose transporters, i.e., *Synechocystis* sp. strain PCC 6714 and *Synechocystis* sp. strain PCC 6803, and expression of *glcP* from *Synechocystis* sp. strain PCC 6803 in the obligate photoautotroph *Synechococcus* sp. strain PCC 7942 results in glucose sensitivity (Joset *et al.*, 1988; Zhang *et al.*, 1998). Our results support this explanation by the requirement for the repressor, FrtR. With the inducer, fructose, expression of *frtABC* in a wild-type *frtR*$^-$ background increased about 30-fold. However, in an *frtR* mutant background, expression was 400-fold higher than that in the wildtype strain. This indicates that in the wild-type strain, under inducing conditions with fructose, *frtABC* was still highly repressed by FrtR. Furthermore, *A. variabilis* strain JU377, which overexpressed *frtABC* in a wild-type *frtR*$^-$ background, was extremely sensitive to fructose. This indicated that overexpression of the fructose transport genes in the presence of FrtR was sufficient to produce a fructose-

sensitive phenotype. Finally, fructose toxicity resulted in impaired phototrophic growth as a function of fructose concentration in strains lacking a functional repressor but not in strains in which fructose uptake was regulated. Together, these findings indicate that fructose uptake must be tightly regulated in order to prevent toxic levels of fructose uptake. The fact that simply overexpressing the fructose transport proteins, even in the absence of fructose, greatly decreased growth suggests that at least part of the problem was the excessive amount of transporters made. However, the addition of fructose to the strains overexpressing the transport proteins resulted in much greater toxicity, which was proportional to the amount of fructose added, indicating that fructose or a metabolic product of fructose was toxic when present in large concentrations in the cell.

For *Anabaena* sp. PCC 7120, which normally cannot take up fructose, the addition of the *frtRABC* genes of *A. variabilis* allowed this strain to use fructose, but only in the dark. In contrast to the case for *A. variabilis*, fructose did not stimulate growth, increase heterocyst frequency, increase cell size, or stimulate nitrogen fixation in *Anabaena* sp. PCC 7120 with the *frtRABC* genes. Excessive entry of fructose into the *Anabaena* sp. strain PCC 7120 mutant with the *frtABC* genes but lacking *frtR* resulted in death. Analysis of the carbon catabolic pathways of the two *Anabaena* strains by use of KEGG, which is based on genome sequences, revealed no obvious differences (Kanehisa *et al.*, 2008); hence, a more detailed metabolomic analysis of fructose metabolism in the two strains may be necessary to understand the mechanism of fructose toxicity.

Although expression of *frtABC* is induced by fructose, I was unable to show that fructose directly affected the binding activity of FrtR to DNA in vitro. Our data suggest that FrtR remained bound to its target sequence irrespective of the presence or absence of

fructose. Thus, either fructose has a low affinity for FrtR or binding of fructose to FrtR has little effect on the affinity of FrtR for DNA. Either condition would make it difficult to detect an FrtR-fructose interaction by our methods. It is also possible that the binding activity of FrtR is modulated by a secondary metabolite of fructose.

In *N. punctiforme,* specialized motile filaments called hormogonia are important in symbiosis (Meeks, 2006) (Meeks *et al.*, 1988). The *hrm* locus plays an important role in repressing further hormogonium differentiation after a functional symbiosis has been established between *N. punctiforme* and its host (Campbell *et al.*, 2003). The homologue of *frtR* in *N. punctiforme*, *hrmR*, has been shown to regulate itself and another gene of unknown function, *hrmE*. The activity of *hrmR* is modulated by an unidentified hormogonium repressing factor that is present in plant extracts (Campbell *et al.*, 2003). Immediately downstream of *hrmE* are the homologs of *frtABC*, namely, *hrmB1*, *hrmB2*, *hrmT*, and *hrmP* (Meeks *et al.*, 2002; Meeks, 2005). It appears likely that *hrmB1-hrmB2-hrmTP*, like *frtABC*, is responsible for fructose transport in *N. punctiforme*. These genes are induced by the hormogonium repressing factor and are thus thought to be part of the *hrm* locus. The close similarity between *frtABC* and *hrmB1-hrmB2-hrmTP* (71 to 85% identity) and the proximity of *hrmB1-hrmB2-hrmTP* to other genes known to be involved in hormogonium formation suggest that fructose or a metabolite thereof might also be involved in regulating hormogonium differentiation. The fructose could be converted to a signaling metabolite that would then provide the signal to repress hormogonium differentiation and establish a lasting relationship with the plant. HrmR is the regulator of *hrmR* and *hrmE* (Campbell *et al.*, 2003), and both of these genes are negatively regulated by fructose (Fig. 4.3). It seems unlikely that HrmR directly regulates *hrmB1-hrmB2-*

*hrmTP* because there is not a putative HrmR binding site upstream of *hrmB1-hrmB2-hrmTP*. A conserved 15-bp regulatory sequence upstream of *hrmB1-hrmB2- hrmTP* that is not bound by HrmR (Meeks and Elhai, 2002) and is absent in the intergenic region between *frtR* and *frtA* in *A. variabilis* might be the regulatory site for another regulatory protein controlling expression of *hrmB1-hrmB2-hrmTP* (Meeks, 2005).

These data and other reports of sugar toxicity in other cyanobacteria (Joset *et al.*, 1988), combined with the apparent inability of the photoautotrophic strain *Anabaena* sp. strain PCC 7120 to use fructose when growing in the light, suggest that strains that are naturally capable of sugar transport and utilization have evolved mechanisms that allow them both to use sugars efficiently and to overcome sugar toxicity. These are of course likely to be metabolically linked processes. *N. punctiforme* and the free-living organism *Anabaena azollae*, which is genetically and morphologically very similar to *A. variabilis*, depend on sugar supplies from a plant when they are in a symbiotic association (Meeks *et al.*, 2002) (Shi and Hall, 1988). In the free-living state, these cyanobacteria retain the ability to use sugars and even show, in modified form, some of the characteristics of symbiosis (Rozen *et al.*, 1986; Rozen *et al.*, 1988), including larger cells, more heterocysts, increased respiration, and increased nitrogen fixation, suggesting that some of the important changes associated with symbiosis are controlled by sugar metabolism in the cyanobacterium rather than by plant-derived factors. *A. variabilis* and *Anabaena* sp. strain PCC 7120 are very similar genetically, sharing about 95% nucleotide identity between homologous genes. They share about 5,000 homologous genes, but *A. variabilis* has about 650 genes that are not present in *Anabaena* sp. strain PCC 7120, and of these, about 240 have homologs in *N. punctiforme* (data calculated from information available

at the Joint Genome Institute (JGI) integrated microbial genome website) (Markowitz *et al.*, 2006). Among these 240 genes, which include the *frtRABC* genes and their homologs in *N. punctiforme*, are likely to be other genes that will provide answers to questions concerning how sugars are used by and may modify important physiological characteristics of true heterotrophic strains. Further system-level analysis, comparing transcriptomes, proteomes, and metabolomes for photoautotrophic versus heterotrophic strain growth with and without sugars, should help to provide answers to these interesting questions.

# Future Directions

## Further studies of the *frtA* and *frtR* promoters.

In *A. variabilis*, both the *frtA* and *frtR* promoters share the same sequences thus both promotes potentially utilize both FrtR binding sites. In *N. punctiforme*, *hmrR* and *hrmE* do not share the same promoter sequences thus they do not share binding sites for HrmR (Appendix 5.5) (Campbell *et al.*, 2003). In *N. punctiforme,* there is a single binding site for HrmR in the *hrmR* promoter and two binding sites for HrmR in the *hrmE* promoter (Appendix 5.5) (Campbell *et al.*, 2003). Furthermore, HrmR binds 4 nucleotides downstream of the *hrmR* transcriptional start site in the *hrmR* promoter, but binds between the -10 and -35 in the *hrmE* promoter (Appendix 5.5). Together, these findings suggest that the interaction between HrmR and its target promoters differs between these two genes. In *A. variabilis*, the role of each binding site in repression of *frtA* and *frtR* is less clear. Although both promoters potentially share the same binding sites, the specific interactions between the two binding sites and the role of these interactions in regulating *frtA* and *frtR* remains unclear.

Further studies on the *frtA* and *frtR* promoters separately can help elucidate the functions of the two FrtR binding sites in regulating these genes. The transcriptional start sites will be determined for both the *frtR* and *frtA* promoters using 5' RACE, which can then be located relative to the FrtR binding sites for each promoter. Additionally, the role of each binding site in regulating *frtA* or *frtR* can be separated by mutating the binding sites individually and simultaneously in *pfrtA* and *pfrtR lacZ* fusions. By comparing the mutant promoter:*lacZ* fusions to wild-type promoter:*lacZ* fusions, in the presence and

absence of fructose, we can determine the specific role of each binding site in regulating *frtA* and *frtR*.

**Further studies in *N. punctiforme.***

No specific plant signal that regulates hormogonia differentiation in *N. punctiforme* has been identified (Campbell *et al.*, 2008; Cohen and Meeks, 1997; Meeks, 2006). The strong similarity between the *frtABC* and *hrmBTP* operons suggests that *hrmBTP* is also a fructose uptake system. Furthermore, these genes are associated with the *hrm* (hormogonia repressing) locus (Meeks, 2006), which suggests that fructose transport may be involved in regulating hormogonia differentiation. Additionally, the high similarity between HrmR and FrtR suggests that HrmR also responds to fructose to regulate hormogonia differentiation. *N. punctiforme* has a well established system for genetic manipulation, which makes it possible to study the importance of fructose transport in regulating hormogonia differentiation directly in this strain.

First we need to verify that fructose transport in *N. punctiforme* is associated with hormogonia regulation. We will make a deletion of the *hrmBTP* genes from the *hrm* locus of *N. punctiforme* to determine whether the *hrmBTP* genes are responsible for fructose transport in *N. punctiforme*. If so, we will also determine whether the mutant can induce or repress hormogonia differentiation in the presence or absence of inducing or repressing plant extracts. If fructose is involved in repressing hormogonia differentiation, then we expect that the *hrmBTP* mutant will continuously differentiate hormogonia in the presence of plant extracts that normally repress hormogonia differentiation; thus, this strain will not form a stable symbiosis with its plant partner.

We will also determine if FrtR and HrmR have similar functions, since our data indicted that HrmR and FrtR exhibit opposite responses to fructose. Since HrmR and FrtR are known to bind the same DNA sequence, we can exchange the coding region of *hrmR* for that of *frtR* in the *N. punctiforme* chromosome to determine whether the two regulators have similar functions in regulating their target genes. The expression of *hrmE* and *hrmR* in response to fructose will be determined by growing the mutant and wild-type strains in the presence and absence of fructose. RT-PCR for the *hrmR* and *hrmE* genes will be done to determine levels of expression in a wild-type and mutant background.

## A fructose-regulated cytochrome oxidase, CoxBAC

*A. variabilis* and *Anabaena* sp. PCC 7120 are very similar strains with no apparent difference between their metabolic pathways; however fructose is able to stimulate growth only in *A. variabilis* (Ungerer *et al.*, 2008). Furthermore, our evidence suggests that *A. variabilis* is able to utilize both fructose and light simultaneously while *Anabaena* sp. PCC 7120 cannot. One explanation for these differences may be the presence of an additional cytochrome c oxidase in *A. variabilis*, *coxBAC*. *coxBAC* is normally expressed constitutively at a low level, however in *A. variabilis* it has an additional transcriptional start site that is stimulated in the presence of fructose (Schmetterer *et al.*, 2001). Additionally, *coxBAC* are essential for chemoheterotrophic growth of *A. variabilis* (Schmetterer *et al.*, 2001). I hypothesize that because *coxBAC* encodes the terminal complex in the respiratory electron transport chain, the increased expression of this cytochrome c oxidase may be essential for increased growth of *A.*

*variabilis* in the presence of fructose. The ability of a mutant in *coxBAC* to grow in the light and with fructose will help to determine the role of these genes in photoheterotrophic growth in *A. variabilis*. Additionally, *coxBAC* can be transferred to *Anabaena* sp. PCC 7120 strain BP291 (which has the *frtRABC* genes), which will allow us to determine if these genes are necessary and sufficient for the increased growth phenotype that we observed in *A. variabilis* grown with fructose.

# SECTION 5 : Appendix

## *TABLE OF STRAINS*

| Strain | Description |
|---|---|
| *A. variabilis FD* | *Anabaena variabilis* parent strain |
| *Anabaena sp. strain PCC 7120* | Wild-type strain |
| *Anabaena sp. strain PCC 7120* Strain BP291 | *frtRABC* expressed from plasmid pBP291 |
| *Anabaena sp. strain PCC 7120* Strain BP292 | *frtABC* expressed from plasmid pBP292 |
| *A. variabilis* BP301 | *frtR* mutated with insert of Nmr cassette at NaeI sites |
| *A. variabilis* BP352 | pBP352 integrated into the chromosome |
| *A. variabilis* BP353 | pBP353 integrated into the chromosome |
| *A. variabilis* BP356 | pBP356 integrated into the chromosome in *ntcA* region via single crossover |
| *A. variabilis* BP457 | pBP457 integrated into the chromosome in *frt* region via single crossover |
| *A. variabilis* BP461 | pBP461 integrated into the chromosome in *frt* region via single crossover |
| *A. variabilis* BP462 | pBP457 integrated into the chromosome in *vnf* region via single crossover |
| *A. variabilis* BP469 | pBP469 integrated into the chromosome in *frt* region via single crossover |
| *A. variabilis* JU336 | pJU336 integrated into the chromosome in *ntcA* region via single crossover |
| *A. variabilis* JU338 | pJU338 integrated into the chromosome in *ntcA* region via single crossover |
| *A. variabilis* JU377 | pJU377 integrated into the chromosome in *ntcA* region via single crossover |
| *A. variabilis* | pJU336 integrated into the chromosome of *A. variabilis* FD BP301 |

| | |
|---|---|
| JU353 | in ntcA region via single crossover |
| *A. variabilis* JU355 | pJU338 integrated into the chromosome of *A. variabilis* FD BP301 in ntcA region via single crossover |
| *Anabaena sp. strain PCC 7120* Strain JU356 | pJU336 integrated into the chromosome in ntcA region via single crossover |
| *Anabaena sp. strain PCC 7120* Strain JU357 | pJU338 integrated into the chromosome in ntcA region via single crossover |
| *A. variabilis* JU333 | pJU333 integrated into the chromosome via double recombination |
| *A. variabilis* JU408 | pJU408 integrated into the chromosome via double recombination |
| *A. variabilis* JU417 | pJU457 integrated into the chromosome via single recombination in *nifH* promoter |
| *A. variabilis* JU420 | pJU397 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU422 | pJU399 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU423 | pJU400 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU424 | pJU401 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU425 | pJU402 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU426 | pJU403 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU427 | pJU404 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU428 | pJU405 integrated into *nif* region via double recombination into strain JU408 |

| | |
|---|---|
| *A. variabilis* JU434 | pJU434 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU435 | pJU435 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU436 | pJU436 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU443 | pJU443 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU444 | pJU444 integrated into *nif* region via double recombination into strain JU408 |
| *A. variabilis* JU453 | pJU453 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU454 | pJU454 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU457 | pJU457 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU466 | pJU466 integrated into *nif* region via double recombination. Created deletion of *nifB* from the chromosome |
| *A. variabilis* JU467 | pJU467 integrated into the chromosome in the *frt* region via single crossover |
| *A. variabilis* JU468 | pJU468 integrated into the chromosome in *nif* region via single crossover |
| *A. variabilis* JU469 | pJU469 integrated into the chromosome in *nif* region of strain JU466 via single crossover |
| *A. variabilis* JU470 | pJU470 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU471 | pJU471 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU472 | pJU472 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* | pJU473 integrated into the chromosome in frt region via single |

| | |
|---|---|
| JU473 | crossover |
| *A. variabilis* JU475 | pJU475 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU476 | pJU476 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU477 | pJU477 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU479 | pJU479 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU480 | pJU480 integrated into the chromosome in frt region via single crossover |
| *A. variabilis* JU483 | pJU480 integrated into the chromosome in nif region via single crossover in nifE promoter |

**PLASMIDS**

| | |
|---|---|
| pAAWY3009 | 9.3-kb library clone of *A. variabilis* DNA containing *frtRABC*; Cmr |
| pAAWY3162 | 9kb library clone of *A. variabilis* DNA containing *Ava_3910-nifH1* |
| pBP288 | Cloning vector for integration of transcriptional fusions into the chromosome; Tcr Kmr Nmr Spr Smr Apr |
| pBP291 | 8.4-kb ScaI/SmaI fragment from pBP289 (containing *frtRABC*) inserted into the SmaI sites of pRL57 |
| pBP292 | 4.9-kb NaeI/SmaI fragment from pBP289 (containing *frtABC*) ligated into the SmaI sites of pRL57 |
| pBP301 | 5-kb BglII fragment of pRL1075 ligated into the BamHI site of pBP299 |
| pBP352 | 5-kb SmaI fragment (containing a *lacZ* Spr Smr cassette) of pBP350 inserted into EcoRV site of pBP351 |
| pBP353 | 5-kb SmaI fragment (containing a *lacZ* Spr Smr cassette) of pBP350 inserted into ClaI site (blunted) of pBP351 |
| pBP356 | PCR-amplified *frtR* gene inserted into SmaI/SacI sites of pBP313 |

| | |
|---|---|
| | under the control of *psbA* promoter |
| pBP457 | PCR fragment using cloned into bglII/smaI of pJU411 |
| pBP459 | PCR fragment using cloned into bglII/smaI of pJU411 |
| pBP461 | PCR fragment using cloned into bglII/smaI of pJU411 |
| pBP469 | PCR fragment using cloned into bglII/smaI of pJU411 |
| pBP485 | PCR fragment using cloned into bglII/smaI of pJU411 |
| pBR322 | Mobilizable plasmid; Apr Tcr |
| pET22B | T7 expression vector; expression induced by IPTG |
| pJU336 | PCR-amplified 400-bp *frtR* promoter fragment inserted into BglII/SmaI sites of pBP288 |
| pJU338 | PCR-amplified 500-bp *frtA* promoter fragment inserted into BglII/SmaI sites of pBP288 |
| pJU375 | Nm cassette from pBP285 (smaI) cloned into pMV2 at EcoRI site. Nm is orientated opposite of the nif genes. |
| pJU776 | pRL2948a cut with smaI and cloned into EcoRV of pJU375. |
| pJU377 | PCR-amplified *frtA* gene inserted into SmaI/SacI sites of pBP313 under the control of *psbA* promoter |
| pJU378 | PCR of pEL1 using the primers nifH69L/nifH69R and self ligated |
| pJU380 | PCR of pEL1 using the primers nifH95L/nifH195R and self ligated |
| pJU381 | PCR of pEL1 using the primers nifH120L/nifH120R and self ligated |
| pJU382 | PCR of pEL1 using the primers nifH170L/nifH170R and self ligated |
| pJU383 | PCR of pEL1 using the primers nifH180L/nifH180R and self ligated |
| pJU384 | PCR of pEL1 using the primers nifHdisRL/nifHdisRR and self ligated |
| pJU385 | PCR of pEL1 using the primers nifHdisHL/nifHdisHR and self ligated |
| pJU386 | PCR of pEL1 using the primers delrpsL/delrpsR and self ligated |
| pJU332 | *nifS-nifD* cloned from pMV2 BamHI into BamHI of pBR322 |

| | |
|---|---|
| pJU333 | *lacZ* from pPE20 cut with kpnI cloned into nifH of pJU332 cut with kpnI |
| pJU362 | 300bp nifH promoter fragment from pBP220 cloned into pBP288 bglII/smaI |
| pJU408 | 377bp AgeI fragment deleted from pJU376 |
| pJU409 | pBP322 cloned into pJU362 HindIII-SphI |
| pJU410 | Nm cassette with terminators at both ends ligated into kpnI of pJU409 |
| pJU411 | PCR fragment of frtABC using frtb-L/frtB-R ligated into HindIII of pJU410 |
| pJU417 | PCR fragment of psbA coding using psbAcodingL /psbAcodingR ligated into HindIII of pJU410 |
| pJU419 | Terminators generated by PCR using term1L/Term2La and term1L/Term2La primers on template pBP285 and cloned into SpeI-EcoRV of pEL1 |
| pJU397 | pRL2948a ligated into pJU378 using scaI. |
| pJU399 | pRL2948a ligated into pJU380 using scaI. |
| pJU400 | pRL2948a ligated into pJU381 using scaI. |
| pJU401 | pRL2948a ligated into pJU382 using scaI. |
| pJU402 | pRL2948a ligated into pJU383 using scaI. |
| pJU403 | pRL2948a ligated into pJU384 using scaI. |
| pJU404 | pRL2948a ligated into pJU385 using scaI. |
| pJU405 | pRL2948a ligated into pJU386 using scaI. |
| pJU429 | PCR fragment of nifB using nifB1L/nifB1R ligated into the SpeI-NaeI of pJU419 to generate nifSU deletion |
| pJU430 | Self ligated PCR fragment of pJU419 using nifHmut1-R/nifHmut1-L |

| | |
|---|---|
| | Self ligated PCR fragment of pJU419 using nifHmut2-R/nifHmut2-L |
| pJU431 | Self ligated PCR fragment of pJU419 using nifHmut3R/nifHmut2-L |
| pJU432 | Self ligated PCR fragment of pJU419 using nifHmut4-R/nifHmut4-L |
| pJU433 | ScaI-nruI fragment of pRL2948a ligated into NaeI of pJU430 |
| pJU434 | ScaI-nruI fragment of pRL2948a ligated into ZraI of pJU419 |
| pJU435 | ScaI-nruI fragment of pRL2948a ligated into ZraI of pJU429 |
| pJU436 | ScaI-nruI fragment of pRL2948a ligated into NaeI of pJU432 |
| pJU443 | ScaI-nruI fragment of pRL2948a ligated into NaeI of pJU433 |
| pJU444 | pBR322 cloned into pAAWY3162 SalI-BamHI |
| pJU445 | PCR fragment using pnifB-L/pnifB-R cloned into bglII/smaI of pJU411 |
| pJU453 | PCR fragment using nifUH-L/nifH1-R2 cloned into bglII/smaI of pJU411 |
| pJU454 | |
| pJU457 | 300bp BglII-SmaI fragment of pBP220 cloned into pJU411 |
| pJU463 | BsrGI-MscI fragment containing nifB deleted from pJU445 |
| pJU466 | BglII fragment containing sacB from pRL2948a cloned into BamHI site on pJU463 |
| pJU468 | PCR fragment using pnifB-L/pnifB-R cloned into bglII/smaI of pJU410 |
| pJU469 | PCR fragment using nifUH-L/nifH1-R2 cloned into bglII/smaI of pJU410 |
| pJU470 | PCR fragment of pEL1 using nifH1-R2/nifUH-L2 cloned into bglII/smaI of pJU411 |
| pJU471 | PCR fragment of pEL1 using nifH1-R2/nifUH-L3 cloned into bglII/smaI of pJU411 |
| pJU472 | PCR fragment of pEL1 using nifH1-R2/nifUH-L4 cloned into bglII/smaI of pJU411 |

| | |
|---|---|
| pJU473 | PCR fragment of pEL1 using nifH1-R2/nifUH-L5 cloned into bglII/smaI of pJU411 |
| pJU475 | PCR fragment of pAAWY3162 using nifSUHL1/nifH1-R2 cloned into bglII/smaI of pJU411 |
| pJU476 | PCR fragment of pAAWY3162 using nifSUHL2/nifH1-R2 cloned into bglII/smaI of pJU411 |
| pJU477 | PCR fragment of pAAWY3162 using nifBSUHL/nifH1-R2 cloned into bglII/smaI of pJU411 |
| pJU479 | PCR fragment of nifE-L2/nifE-R10 using cloned into bglII/smaI of pJU411 |
| pJU480 | PCR fragment of nifE-L3/nifE-R10 using cloned into bglII/smaI of pJU411 |
| pRL2948a | Source of mobilization site, *oriT*, and *sacB* gene, which confers sucrose sensitivity; Cmr Emr |

## *TABLE OF PRIMERS*

| Primer | Sequence |
|---|---|
| fruR-L3 | 5’ – ATTCATATGCTTTTTATCTTTGCACACCTGTGCAT – 3’ |
| fruR-R3 | 5’ - ATTGGATCCTCATCATACTGCGTAAGTCCTAGTACTATCTCTTTTGA – 3’ |
| nifUH-L | 5’ - AATAGATCTAGCCCAAGAACAAACATTG – 3’ |
| nifH1-RPE | 5’ - TGGTTAGTTGGTCGGTCTTTG – 3’ |
| nifH1-R2 | 5’ - ATACCCGGGTCTAATGTTTTCGTCAGTCA – 3’ |
| frtR131-L | 5’ - GAAGATCTGAAAGACTTTTTCATATTTGGTTATG – 3’ |
| frtR-R10 | 5’ - ATACCCGGGTGCTTTTCGAGCAATATCTTCA – 3’ |
| frtR-R | 5’ - GGGGTGGGGTAATATAACTGCTCA – 3’ |
| frtR-L | 5’ - GACGGTATCTTGGTAGCGGACTCA – 3’ |
| frtA-R | 5’ - GCGGCCCGTTGACTATGACTAC – 3’ |
| frtA333 | 5’ - GAAGATCTGATAAAAAGCATAGGTTATGCACACCT – 3’ |
| nifH302L | 5’ - GAAGATCTCAGCCTAGTAGTAGAAGCAGTT – 3’ |
| frtA498 | 5’ - GAAGATCTATTAGGGGACTGTCTCGTAAAG – 3’ |
| frtR397 | 5’ - GAAGATCTACGCGTAGCAGCTTGTCTTT – 3’ |
| nifS-L | 5’ - AAGGTAGATCCAGAGGTTGTAGAGG – 3’ |
| NmkpnL | 5’ - GATCTCAATGAATATTGGTTGAC – 3’ |
| nifSU-L | 5’ - AATAGTACTAGAGATTGCCCAACACCAC – 3’ |
| nifSU-R | 5’ - AATAAGCTTGTAGGTTTACTTCCGCCTTTATTG – 3’ |
| Nm5'termL1 | 5’ - ATAGGTACCAATTCAAAAACGGGCAGACATGGCCTGCCCGGTTAGGATCTCAATGAATATTGGTTGAC – 3’ |
| Nm5'termL2 | 5’ - ATAGGTACCAATTCAAAAACGGGCAGACATGGCCTGCCCGGTTAGGATCTCAATGAATATTGGTTGACACGGGCGTATAA – 3’ |
| Nm5'termR | 5’ - ATAGGTACCGATGGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCGAA – 3’ |
| term1L | 5’ - AATACTAGTGCATCGCCTTCTATCGCCTTC – 3’ |
| term2L | 5’ - AATAAGCTTGGATGTGCTGCAAGGCGATTAA – 3’ |
| Term2La | 5’ - AATAAGCTTAGGCTTTACACTTTATGCTTCCGGCTC – 3’ |
| Term2Ra | 5’ - AATGATATCAGGCTTTACACTTTATGCTTCCGGCTC – 3’ |
| FrtA-RPE | 5’ - TTGGGCCATTGGGAGAAATG – 3’ |
| FrtA-R10 | 5’ - ATACCCGGGACGCGTAGCAGCTTGTCTTT – 3’ |
| FrtR397-L | 5’ - GAAGATCTACGCGTAGCAGCTTGTCTTT – 3’ |
| nifB1L | 5’ - AATGTCGACAACAAGATGATTCGGGAACAAGGTGCATTC – 3’ |
| nifB1R | 5’ - AATACTAGTCGGTTTCGTTGTTCGCATACATAATTGTCA – 3’ |
| nifB1RTL | 5’ - GGCAGCTAGTCCACCGACAT – 3’ |
| nifB1RTR | 5’ - ATCCGCAACCACCTGATTTT – 3’ |

| | |
|---|---|
| nifH1RTL | 5’ - ACAGGCGTGAGATCCAAACA – 3’ |
| nifH1RTR | 5’ - CATCAAACGGGTGGAGTCAG – 3’ |
| nifK1RTL | 5’ - CTACCTTGAGGAGGAGTGAA – 3’ |
| nifK1RTR | 5’ - CTCGGTGTATTCTGGCTGTT – 3’ |
| UH-L3 | 5’ - CAGCCTAGTAGTAGAAGCAGTTTAG – 3’ |
| UH-R3 | 5’ - GTTTGGATCTCACGCCTGTTG – 3’ |
| UH-L4 | 5’ - GACTATGGACTCAGCACTCATCAC – 3’ |
| nifU-RTL | 5’ - GCAACAAACCTCAACAATAAAGG – 3’ |
| nifU-RTR | 5’ - GGTCTTACTTCTTCGTCTAATACTTTTTG – 3’ |
| nifSUHL1 | 5’ -TGAAGATCTAAGGTAGATCCAGAGGTTGTAGAGG – 3’ |
| nifSUHL2 | 5’ - TGAAGATCTAGCAATGGAATAAGGGCTAATGAG – 3’ |
| nifBSUHL | 5’ - TGAAGATCTAGCAACCGCGTCTGATAGTGT – 3’ |
| nifSRTL | 5’ - CATTCGTGCCGCATTGTTAGCC – 3’ |
| nifSRTR | 5’ - ACAGTCCCGGTTTCGTTGTTCG – 3’ |
| nifBRTR2 | 5’ - GCAATACGTTCTTGGAGCTTTTC – 3’ |
| nifUH-L2 | 5’ - AATAGATCTGGGAGTCATTGAAGATAACG – 3’ |
| nifUH-L3 | 5’ - AATAGATCTCTGACTTTAGATGAAGCCCTG – 3’ |
| nifUH-L4 | 5’ - AATAGATCTGAAAATAAGGTACGTCGCATAG – 3’ |
| nifUH-L5 | 5’ - AATAGATCTGGCAAAAACGACCCCTC – 3’ |
| UH-L5 | 5’ - GGCGACGTTCTACAAACCC – 3’ |
| nifHmut5-R | 5’ - AATAGATCTCATCACTTAATTACATAACGAACCCATCATGAACAATAATTCTACCG – 3’ |
| nifHmut5-L | 5’ - AATAGATCTCTAATGACTAAACTGCTTCTACTACTAGGCTGGGATTAATGCG – 3’ |
| nifHmut6-R | 5’ - AATAGATCTTCAATGCTATGGACTATGGACTATGGACTCAGC – 3’ |
| nifHmut6-L | 5’ - AATAGATCTCACTGTTGACTAATGACCATTGACTAATGACTAATGACTAATGACTAAACT |
| nifHmut7-R | 5’ - AATAGATCTCTGTAGAGCGATCGCCCCCTCTTC – 3’ |
| nifHmut7-L | 5’ - AATAGATCTCCGGTAGAATTATTGTTCATGATGGGTTCGTTATG |
| nifHmut9-R | 5’ - AATAGATCTACCGGTTTTTCTGTAGAGCGATCGCCC – 3’ |
| nifHmut9-L | 5’ - AATAGATCTTTGTTCATGATGGGTTCGTTATGTAATTAAGTGATGAGTG – 3’ |
| nifBRT1 | 5’ - CTATAAAGTCCTTGGTTTTGGCT – 3’ |
| nifBRT2 | 5’ - GCTGAGTAGGGCAGGCTATAAAGT – 3’ |
| nifBPCR1 | 5’ - GGTAGAATGTGTTTACAGCCAAG – 3’ |
| nifBPCR2 | 5’ - GAATGTGTTTACAGCCAAGCCAG – 3’ |
| nifERT1 | 5’ - CGACGTTGTTAATTGTCAGTTATC – 3’ |
| nifERT2 | 5’ - GAAATAATCACCCAATTCACCGATA – 3’ |

| | |
|---|---|
| nifEPCR1 | 5’ - GATAACCATTCATAATAAACTTCTTGCA – 3’ |
| nifEPCR2 | 5’ - TCTTCTTTCATCACGCGAAATAC – 3’ |
| nifEPCR3 | 5’ - AATCCTTTCCCTAGCCCCT – 3’ |
| frtXchkR | 5’ - TGCGGAGTCTGTCAATCATG – 3’ |
| XchkL | 5’ - CTACGATACGGGAGGGCTTAC – 3’ |
| XchkR | 5’ - TTGTTGAGAACGGCGATGA – 3’ |
| NifHdisH-L | 5’ - GCTATCGATATATATTATGGACTCAGCACTCATCACTTAATTACA – 3’ |
| NifHdisH-R | 5’ - GCTATCGATTCTCTCTGATTTTTCACTGTTGACTAATGACCATTG – 3’ |
| NifHdisR-L | 5’ –GCTATCGATAAAAATTAGTCAATGGTCATTAGTCAACAGTG – 3’ |
| NifHdirR-R | 5’ - GCTATCGATTTTGACTAATGACTAATGACTAAACTGCTTC – 3’ |
| nifH180-R | 5’ – GCTATCGATGTACAGTGCTGAGTCCATAGTCCATA – 3’ |
| nifH180-L | 5’ - GCTATCGATTTACATAACGAACCCATCATG – 3’ |
| nifH170-R | 5’ - GCTATCGATGTGATGAGTGCTGAGTCCATA – 3’ |
| nifH170-L | 5’ - GCTATCGATTAACGAACCCATCATGAACA – 3’ |
| nifH120-R | 5’ - GCTATCGATCAGAAAAACCGGTAGAATTATT – 3’ |
| nifH120-L | 5’ - GCTATCGATCAGAAAAACCGGTAGAATTATT – 3’ |
| nifH95-R | 5’ - GCTATCGATGCCGAAGAGGGGGCGATCGCTC – 3’ |
| nifH95-L | 5’ - GCTATCGATCAAACCCCTCACAGCCATAGC – 3’ |
| nifH82-R | 5’ - GCTATCGATTTTGTAGAACGTCGCCGAAGA – 3’ |
| nifH82-L | 5’ - GCTATCGATCCATAGCTCAACAGGCGTGAGA – 3’ |
| nifH69-R | 5’ - GCTATCGATCTATGGCTGTGAGGGGTTTGTAG – 3’ |
| nifH69-L | 5’ - GCTATCGATCGTGAGATCCAAACACAAAGAC – 3’ |
| vnfHBgal-L45 | 5’ - GAAGATCTTGCATCAATCAAGATATGATTTAGTGATT – 3’ |
| vnfHBgal-L1380 | 5’ - GAAGATCTCAGAACGCGCTTAGGGATGAG – 3’ |
| vnfHBgal-L1961 | 5’ - GAAGATCTAAGACGTTTTCATTGTTTGG – 3’ |
| vnfHBgal-R1 | 5’ - ATACCCGGGATCGCGGAAGCCTTTGAGTACTACTT – 3’ |

## 5.3 Alignments

**Alignment** *nifH1* upstream region of four *Anabaena* strains

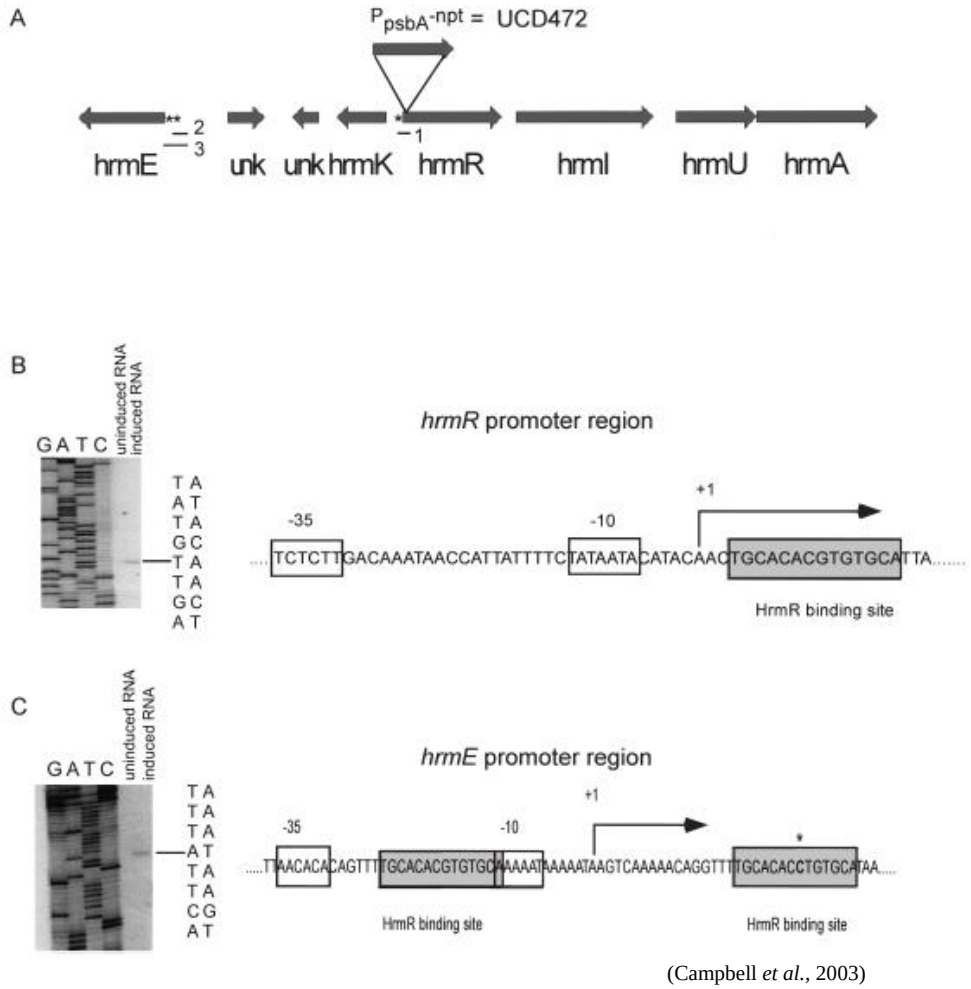

Appendix **5.3** Alignment of the *nifU-H* intergenic of four related strains of *Anabaena*. *nifH* coding region is in red. Boxes surround conserved regions between these for strains that were mutated as indicated in Figure 3.1.

# 5.4 Alignment of the nifU-H intergenic region

```
7120            CATTCAAAAAGTATTAGACGAAGAAGTAAGACCCGTATTGATTGCCGACGGCGGAGATGT
Avar            CATTCAAAAAGTGTTAGACGCAGAAGTAAGACCCGTATTGATTGCCGACGGCGGAGATGT
                *********** ******* ************************************

7120            ACTCTACGATGTAGACGGCGATATTGTCAAAGTAGTACTGCAAGGCGCGTGTGGCTCCTG
Avar            ACTCTACGATGTAGACGGCGATATTGTCAAAGATGTACTGCAAGGCGAGTGTGGCTCCTG
                ********************************  ************ ************

7120            TTCTAGTAGTACAGCCACCTTAAAGATAGCGATTGAATCCAGATTACGCGATCGCATCAA
Avar            TTCTAGTAGTACAGCCACCTTGAAGATAGCGATTGAATCCAGATTACGCGATCGCATTAA
                ********************* ********************************** **
                                                ↓--------------------------
7120            CCCCAGCCTAGTAGTAGAAGCAGTTTAGTTAACAGTTAACAGTAA----------ACCGT
Avar            TCCCAGCCTAGTAGTAGAAGCAGTTTAGTCATTAGTCATTAGTCATTAGTCAATGGTCAT
                 **************************** *  *** *  *** *           * *
                --------↓↓-------------------------------------------------
7120            TAACTAACACCCAAAA------------------------GAACTT--------TCAC-
Avar            TAGTCAACAGTGAAAAATCAATGCTATGGACTATGGACTATGGACTCAGCACTCATCACT
                **   ****   ****                          * ***         ****
                -------↓
7120            -AACTACATAACGAACCCATCATGAACACTAATTCTACTGGTTTTTCTGTGGAGCGATCG
Avar            TAATTACATAACGAACCCATCATGAACAATAATTCTACCGGTTTTTCTGTAGAGCGATCG
                 ** ********************** ********* *********** *********

7120            CCCCCTCTTCGGCGACGTTCTACAAACCCCTCACAGCCATAGCTCAACAGGCGTGAGATC
Avar            CCCCCTCTTCGGCGACGTTCTACAAACCCCTCACAGCCATAGCTCAACAGGCGTGAGATC
                ************************************************************

7120            CAAACACAAAGACCGACCAACTAACCAACCAATTGCAGGAAAAGAGAACAATGACTGACG
Avar            CAAACACAAAGACCGACCAACTAACCAACCAATTGCAGGAAAAGAGAACAATGACTGACG
                ************************************************************
```

Appendix **5.4** Alignment of the *nifU-H* intergenic region. Sequences in red and blue match the stem-loop structures in Figure 3.11. The deletion in JU434 that did not affect nitrogenase activity has a green line over it. The deletion in JU437 that abolished nitrogenase activity has a pink line over it. The start and stop codons of *nifU* and *nifH* are highlighted yellow.

# 5.5 hrm locus in N. punctiforme




(Campbell *et al.*, 2003)

Appendix **5.5.** (A) Map of open reading frames in the *hrm* locus of *N. punctiforme*. Arrows indicate the direction of transcription. (B) and (C) identification of the transcript 5' ends of the *hrmR* and *hrmE* genes and locations of the putative promoter regions with HrmR binding sites. The HrmR binding sites deduced from EMSA are shown relative to the putative transcriptional start points with a *. The -10 and -35 boxes drawn were drawn based on the location of each putative transcript start site and not on any specific knowledge of sequences recognized by a *N. punctiforme* sigma factor; in cyanobacteria, the -35 box in particular could have various hypothetical designations.

# 5.6 NtcA binding sites in *Anabaena*

A

| | |
|---|---|
| Consensus NtcA site | **TGTA** N8 **TACA** |
| *glnA* NtcA site | **TGTA**GTCGGGGT**TACA** |
| *ntcA* NtcA site | **AGTA**TAGGAAAG**TACA** |
| *ccbP* NtcA | **GTTC**TGAGTGGT**CACA** |
| *A. variabilis nifH1* | **TCAT**CACTTAAT**TACA** |
| *A. 7120 nifH* | **AACT**TTCACAAC**TACA** |
| JU424 *nifH* | **TCAT**TGCACATC**CGAT** |
| JU425 *nifH* | **TGTA**TGCACATC**TACA** |

B

| | Numbers of the nucleotides at each position[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nucleotide | 1L | 2L | 3L | 4L | 5L | 6L | 7L | 8L | 8R | 7R | 6R | 5R | 4R | 3R | 2R | 1R |
| A | 3 | 0 | 0 | 10 | 3 | 0 | 7 | 4 | 1 | 1 | 3 | 2 | 1 | 10 | 0 | 8 |
| C | 2 | 0 | 0 | 0 | 0 | 8 | 1 | 0 | 4 | 2 | 0 | 4 | 0 | 0 | 11 | 1 |
| G | 1 | 11 | 0 | 1 | 4 | 1 | 0 | 4 | 0 | 2 | 6 | 1 | 0 | 1 | 0 | 0 |
| T | 5 | 0 | 11 | 0 | 4 | 2 | 3 | 3 | 5 | 6 | 2 | 4 | 10 | 0 | 0 | 1 |
| Consensus | N | **G** | **T** | **A** | N | C | A | N | N | T | G | N | **T** | **A** | **C** | **A** |

Appendix 5.6 Comparison of NtcA binding sites in *Anabaena*. (A) Comparison of several NtcA binding sites to the mutant NtcA binding sites created upstream of *nifH1*. *glnA*, *ntcA*, *ccbP*, and *nifH* binding sites have been experimentally validated using EMSA. (B) Broader comparison of NtcA binding sites in *Anabaena*.

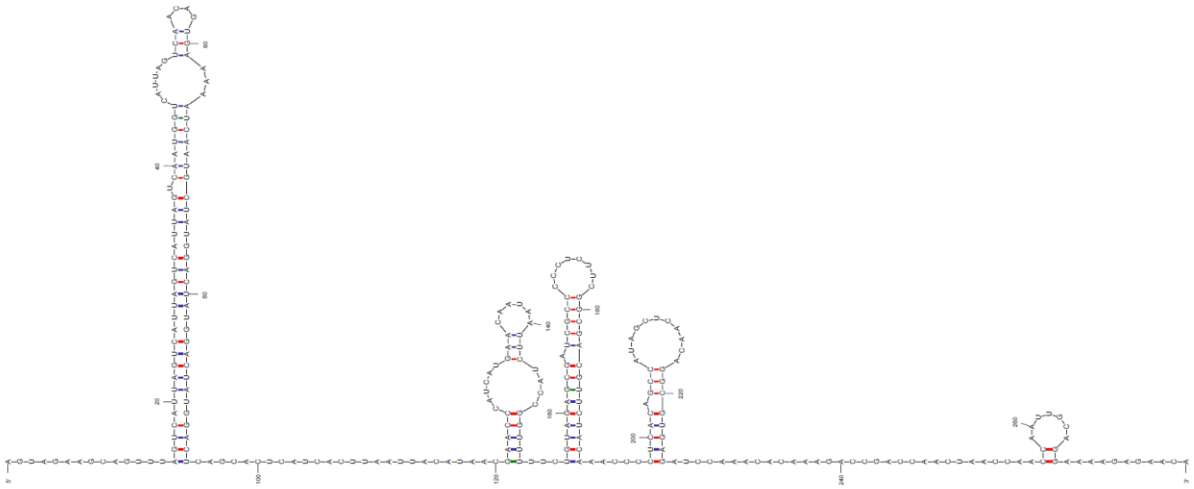
# 5.7 FurA binding site

```
7120            ACTCTACGATGTAGACGGCGATATTGTCAAAGTAGTACTGCAAGGCGCGTGTGGCTCCTG
Avar            ACTCTACGATGTAGACGGCGATATTGTCAAAGATGTACTGCAAGGCGAGTGTGGCTCCTG
                ********************************  ************ ************

7120            TTCTAGTAGTACAGCCACCTTAAAGATAGCGATTGAATCCAGATTACGCGATCGCATCAA
Avar            TTCTAGTAGTACAGCCACCTTGAAGATAGCGATTGAATCCAGATTACGCGATCGCATTAA
                ******************** ********************************** **

7120            CCCCAGCCTAGTAGTAGAAGCAGTTTAGTTAACAGTTAACAGTAA----------ACCGT
Avar            TCCCAGCCTAGTAGTAGAAGCAGTTTAGTCATTAGTCATTAGTCATTAGTCAATGGTCAT
                 **************************** *  *** *  *** *            * *

7120            TAACTAACACCCAAAA------------------------GAACTT--------TCAC-
Avar            TAGTCAACAGTGAAAAATCAATGCTATGGACTATGGACTATGGACTCAGCACTCATCACT
                **   ****   ****                          * ***         ****

7120            -AACTACATAACGAACCCATCATGAACACTAATTCTACTGGTTTTTCTGTGGAGCGATCG
Avar            TAATTACATAACGAACCCATCATGAACAATAATTCTACCGGTTTTTCTGTAGAGCGATCG
                 ** ********************** ********* *********** *********

7120            CCCCCTCTTCGGCGACGTTCTACAAACCCCTCACAGCCATAGCTCAACAGGCGTGAGATC
Avar            CCCCCTCTTCGGCGACGTTCTACAAACCCCTCACAGCCATAGCTCAACAGGCGTGAGATC
                ************************************************************

7120            CAAACACAAAGACCGACCAACTAACCAACCAATTGCAGGAAAAGAGAACAATGACTGACG
Avar            CAAACACAAAGACCGACCAACTAACCAACCAATTGCAGGAAAAGAGAACAATGACTGACG
                ************************************************************
```

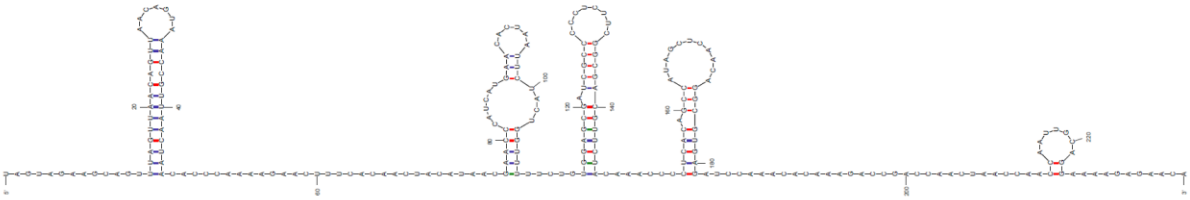
Appendix 5.7 Comparison of FurA binding sites in *Anabaena 7120* and *Anabaena variabilis.* FurA binding site is shown in blue for *A. 7120* compared to predicted FurA binding site in *A. variabilis. nifU* and *nifH* stop and start codons are highlighted yellow. The 5' half of the FurA binding site is identical in both strains. The 3' region of the binding site is composed of a pair of repeates that differs between the two strains, TTAACAG in *A. 7120* and TCATTAG in *A. variabilis*. The sequence is repeated twice in *A. 7120* but is repeated 5 times in *A. variabilis*. FurA is known to oligimerize along DNA, thus we hypnotize that if it binds the first two repeates in *A. variabilis* it is also likely to bind the next three.

# 5.8 Comparison of Secondary structure in the *nifU-H* intergenic region of *A. variabilis* and *A. 7120*.

***Anabaena variabilis***



***Anabaena* spp. *7120***



Appendix 5.8 A comparison of potiential secondary structure in the *nifU-H* intergenic region in *A. variabilis* and *A. 7120*.

# SECTION 6 : REFERENCES

Adams, D. G. (2000). "Heterocyst formation in cyanobacteria." Curr Opin Microbiol **3**(6): 618-24.

Adams, D. G. and N. G. Carr (1989). "Control of heterocyst development in the cyanobacterium *Anabaena cylindrica*." J of Gen Microbiol **135**: 839-849.

Adams, D. G. and P. S. Duggan (2008). "Cyanobacteria-bryophyte symbioses." J Exp Bot **59**(5): 1047-58.

Agar, J. N., P. Yuvaniyama, et al. (2000). "Modular organization and identification of a mononuclear iron-binding site within the NifU protein." J Biol Inorg Chem **5**(2): 167-177.

Aldea, M. R., Mella-Herrera, Golden, J. (2007). "Sigma factor genes *sigC*, *sigE*, and *sigG* are upregulated in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120." J. Bacteriol. **189**(22): 8392-8396.

Alifano, P., F. Rivellini, et al. (1994). "Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA." Gen Dev **8**(24): 3021-3031.

Allen, M. B. and D. I. Arnon (1955). "Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm." Plant Physiol **30**: 366-372.

Anderson, S. L. and L. McIntosh (1991). "Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process." J Bacteriol **173**: 2761-2767.

Bauersachs, T., E. C. Hopmans, et al. (2009). "Rapid analysis of long-chain glycolipids in heterocystous cyanobacteria using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry." Rapid Communications in Mass Spectrometry **23**(9): 1387-1394.

Bensing, B. A., Meyer, et al. (1996). "Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-

induced plasmid transfer system of *Enterococcus faecalis*." Proc Nat Aca Sci USA **93**(15): 7794-7799.

Böhme, H. (1998). Regulation of nitrogen fixation in heterocyst-forming cyanobacteria. Trends Plant Sci. 3:346-351.

Bolin, J. T. Muchmore, W.; Minor, W.; Morgan, T. V.; Mortenson, L. E. In New Horizons in Nitrogen Fixation; Palacios, R., Mora, J., Newton, W. E., Eds.; Kluwer Academic Publishers: Dordrecht, 1993; p 89.

Borthakur, P. Orozco, et al. (2005). "Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120." Mol Microbiol **57**(1): 111-23.

Boussiba, S. and J. Gibson (1991). "Ammonia translocation in cyanobacteria." FEMS Microbiol Let **88**(1): 1-14.

Braun-Howland, E. B., L. P., et al. (1988). "Dinitrogenase reductase of nitrogenase in the cyanobacterial symbionts of three *Azolla* species: localization and sequence of appearance during heterocyst differentiation." Planta **176**: 319-332.

Bricker, A. L. and J. G. Belasco (1999). "Importance of a 5' Stem-Loop for Longevity of pa*p*A mRNA in *Escherichia coli*." J. Bacteriol. **181**(11): 3587-3590.

Brigle, K. E., M. C. Weiss, et al. (1987). "Products of the iron-molybdenum cofactor-specific biosynthetic genes, *nifE* and *nifN*, are structurally homologous to the products of the nitrogenase molybdenum-iron protein genes, *nifD* and *nifK*." J. Bacteriol. **169**(4): 1547-1553.

Brusca, J. S., C. J. Chastain, et al. (1990). "Expression of the *Anabaena* sp. strain PCC 7120 *xisA* gene from a heterologous promoter results in excision of the *nifD* element." J Bacteriol **172**(7): 3925-31.

Brusca, J. S., M. A. Hale, et al. (1989). "Excision of an 11-kilobase-pair DNA element from within the *nifD* gene in *Anabaena variabilis* heterocysts." J Bacteriol **171**(8): 4138-45.

Burgess, B. K. and D. J. Lowe (1996). "Mechanism of Molybdenum Nitrogenase." Chemical Reviews **96**(7): 2983-3012.

Burris, R. H. and G. P. Roberts (1993). "Biological nitrogen fixation." Annu Rev Nutr **13**: 317-35.

Cai, Y. and C. P. Wolk (1990). "Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences." J. Bacteriol. **172**: 3138-3145.

Campbell, E. L., H. Christman, et al. (2008). "DNA Microarray Comparisons of Plant Factor- and Nitrogen Deprivation-Induced Hormogonia Reveal Decision-Making Transcriptional Regulation Patterns in *Nostoc punctiforme* " J. Bacteriol. **190**(22): 7382-7391.

Campbell, E. L., Wong, et al. (2003). "DNA binding properties of the HrmR protein of *Nostoc punctiforme* responsible for transcriptional regulation of genes involved in the differentiation of hormogonia." Mol Microbiol **47**(2): 573-82.

Carrasco, C. D., Buettner, et al. (1995). "Programmed DNA rearrangement of a cyanobacterial *hupL* gene in heterocysts." Proc Natl Acad Sci U S A **92**(3): 791-5.

Carrasco, C. D., Garcia, et al., Eds. (1998). Programed DNA rearrangement of a hydrogenase gene during *Anabaena* heterocyst development. BioHydrogen. New York, Plenum Press.

Carrasco, C. D., Ramaswamy, et al. (1994). "*Anabaena xisF* gene encodes a developmentally regulated site-specific recombinase." Genes Dev **8**(1): 74-83.

Carrasco, C. D. (1995). "Characterization of the recombinase gene required for the *hupl* (sic) DNA rearrangement in *Anabaena* sp. strain PCC 7120. Abstr. Vth Cyanobacterial Molec. Biol. Workshop, Pacific Grove, CA, p. 26."

Chai, W. and V. Stewart (1998). "NasR, a novel RNA-binding protein, mediates nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* m5al nasF operon leader in Vitro." J Mol Biol **283**(2): 339-351.

Chan, M. K., J. Kim, et al. (1993). "The nitrogenase FeMo-cofactor and P-cluster pair: 2.2 A resolution structures." Science **260**(5109): 792-4.

Chastain, C. J., Brusca, et al. (1990). "A sequence-specific DNA-binding factor (VF1) from *Anabaena* sp. strain PCC 7120 vegetative cells binds to three adjacent sites in the *xisA* upstream region." J Bacteriol **172**(9): 5044-51.

Chen, C.-Y. A., Beatty, et al. (1988). "An intercistronic stem-loop structure functions as an mRNA decay terminator necessary but insufficient for *puf* mRNA stability." Cell **52**(4): 609-619.

Cohen, M. F. and J. C. Meeks (1997). "A Hormogonium Regulating Locus, *hrmUA*, of the Cyanobacterium Nostoc punctiforme Strain ATCC 29133 and its Response to an Extract of a Symbiotic Plant Partner *Anthoceros punctatus*" Mol Plant-Microbe Interactions **10**(2): 280-289.

Corbett, M. C., Y. Hu, et al. (2006). "Structural insights into a protein-bound iron-molybdenum cofactor precursor." Proc Nat Acad Sci USA **103**(5): 1238-1243.

Cumino, A. C., C. Marcozzi, et al. (2007). "Carbon Cycling in *Anabaena* sp. PCC 7120. Sucrose Synthesis in the Heterocysts and Possible Role in Nitrogen Fixation." Plant Physiol. **143**(3): 1385-1397.

Curatti, L., J. A. Hernandez, et al. (2007). "In vitro synthesis of the iron molybdenum cofactor of nitrogenase from iron, sulfur, molybdenum, and homocitrate using purified proteins." Proc Nat Acad Sci USA **104**(45): 17626-17631.

Currier, T. C. and C. P. Wolk (1979). "Characteristics of *Anabaena variabilis* influencing plaque formation by cyanophage N-1." J Bacteriol **139**(1): 88-92.

Davis, C. S., D.J. (2006). "Transalantic abundance of the N2 fixing colonial cyanobacterium *Trichodesimum*." Science(312): 1517-1520.

Dingler, C. and J. Oelze (1985). "Reversible and irreversible inactivation of cellular nitrogenase upon oxygen stress in *Azotobacter vinelandii* growing in oxygen controlled continuous culture." Arch Microbiol **141**(1): 80-4.

Dingler, C. and J. Oelze (1987). "Superoxide dismutase and catalase in *Azotobacter vinelandii* grown in continuous culture at different dissolved oxygen concentrations." Arch of Microbiol **147**(3): 291-294.

Dominic, B., Y.-B. Chen, et al. (1998). "Cloning and transcriptional analysis of the *nifUHDK* genes of *Trichodesmium* sp. IMS101 reveals stable *nifD, nifDK* and *nifK* transcripts." Microbiol **144**(12): 3359-3368.

Dong, U., X. Huang, X.-Y. Wu, and J. Zhao. (2000). "Identification of the active site of HetR protease and its requirement for heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bacteriol 182:1575-1579."

Dos Santos, P. C., Johnson, et al. (2007). "Controlled Expression of *nif* and *isc* Iron-Sulfur Protein Maturation Components Reveals Target Specificity and Limited Functional Replacement between the Two Systems ." J. Bacteriol. **189**(7): 2854-2862.

Ehira, S., M. Ohmori, et al. (2003). "Genome-wide expression analysis of the responses to nitrogen deprivation in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120." DNA Res **10**(3): 97-113.

Einsle, O., F. A. Tezcan, et al. (2002). "Nitrogenase MoFe-Protein at 1.16 A Resolution: A Central Ligand in the FeMo-Cofactor " Science **297**(5587): 1696-1700.

Elfgren, I. K. (2003). "Studies on the Life Cycles of Akinete Forming Cyanobacteria." Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology **856**.

Elhai, J. and C. P. Wolk (1988). "Conjugal transfer of DNA to cyanobacteria." Methods Enzymol. **167**: 747-754.

Elhai, J. and C. P. Wolk (1988). "A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers." Gene **68**(1): 119-138.

Emerich, D. W. and R. H. Burris (1978). "Complementary functioning of the component proteins of nitrogenase from several bacteria." J Bacteriol **134**(3): 936-43.

Emory, S. A., P. Bouvet, et al. (1992). "A 5'-terminal stem-loop structure can stabilize mRNA in Escherichia coli." Gen Dev **6**(1): 135-148.

Enkh-Amgalan, J., H. Kawasaki, et al. (2006). "Cloning and characterization of a novel gene involved in nitrogen fixation in *Heliobacterium chlorum*: a possible regulatory gene." Arch of Microbiol **186**(4): 327-337.

Fan, Q., G. Huang, et al. (2005). "Clustered genes required for synthesis and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120." Mol Microbiol **58**(1): 227-243.

Fay, P. (1970). "Photostimulation of nitrogen fixation in *Anabaena cylindrica*." Biochim Biophys Acta **216**(2): 353-356.

Fay, P. (1976). "Factors influencing dark nitrogen fixation in a blue-green alga." Appl. Environ. Microbiol. **31**(3): 376-379.

Fay, P. (1992). "Oxygen relations of nitrogen fixation in cyanobacteria." Microbiol Rev **56**: 340-373.

Fay, P. and A. E. Walsby (1966). "Metabolic activities of isolated heterocysts of the blue-green alga *Anabaena cylindrica*." Nature **209**(18): 94-95.

Fiedler, G., M. Arnold, et al. (1998). "The DevBCA exporter is essential for envelope formation in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120." Mol Microbiol **27**(6): 1193-202.

Fiedler, G., A. M. Muro-Pastor, et al. (2001). "NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-specific ATP-binding cassette transporter in *Anabaena* spp." J. Bacteriol. **183**(12): 3795-9.

Fleming, H. and R. Haselkorn (1974). "The program of protein synthesis during heterocyst differentiation in nitrogen-fixing blue-green algae." Cell **3**: 159-170.

Flores, A. H. a. E., Ed. (January 2008). The Cyanobacteria: Molecular Biology, Genomics and Evolution. The Cyanobacteria: Molecular Biology, Genomics and Evolution, Caister Academic Press.

Flores, E. and A. Herrero (2005). "Nitrogen assimilation and nitrogen control in cyanobacteria." Biochem Soc Trans **33**(Pt 1): 164-7.

Flores, E., A. Herrero, et al. (2006). "Is the periplasm continuous in filamentous multicellular cyanobacteria?" Trends Microbiol **14**(10): 439-43**.**

Flores, E. and G. Schmetterer (1986). "Interaction of fructose with the glucose permease of the cyanobacterium *Synechocystis* sp. strain PCC 6803." J Bacteriol **166**(2): 693-6.

Forchhammer, K. and N. Tandeau de Marsac (1995). "Functional analysis of the phosphoprotein PII (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942." J. Bacteriol. **177**(8): 2033-2040.

Franche, C. and G. Cohen-Bazire (1987). "Evolutionary divergence in the *nifH.D.K*. gene region among nine symbiotic *Anabaena azollae* and between *Anabaena azollae* and some free-living heterocystous cyanobacteria." Symbiosis **3**: 159-178.

Frías, J. E., E. Flores, et al. (1997). "Nitrate assimilation gene cluster from the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120." J. Bacteriol. **179**: 477-486.

Fu W, J. R., Morgan TV, Dean DR, Johnson MK (1994). "*nifU* gene product from *Azotobacter vinelandii* is a homodimer that contains two identical [2Fe-2S] clusters." Biochem **33**: 13455–63.

Gambacorta, A., Romano, I., Trincone, A., Soriente, A., Giordano, M. and Sodano, G., (1996). "The heterocyst glycolipids of cyanobacteria. 5. Heterocyst glycolipids from five nitrogen-fixing cyanobacteria." Gazz. Chim. Ital. **126**: 653-656.

Gay, P., D. Le Coq, et al. (1985). "Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria." J. Bacteriol. **164**(2): 918-921.

Gebhardt, J. S. and S. A. Nierzwicki-Bauer (1991). "Identification of a common cyanobacterial symbiont associated with *Azolla* spp. through molecular and morphological characterization of free-living and symbiotic cyanobacteria." Appl Environ Microbiol **57**(8): 2141-6.

George, S. J., R. Y. Igarashi, et al. (2007). "Identification of a Mo Fe S Cluster on NifEN by Mo K-Edge Extended X-ray Absorption Fine Structure." J Am Chem Soc **129**(11): 3060-3061.

Golden, J. W., C. D. Carrasco, et al. (1988). "Deletion of a 55-kilobase-pair DNA element from the chromosome during heterocyst differentiation of *Anabaena* sp. strain PCC 7120." J Bacteriol **170**(11): 5034-41.

Golden, J. W., L. L. Whorff, et al. (1991). "Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120." J Bacteriol **173**(22): 7098-105.

Golden, J. W. and H. S. Yoon (2003). "Heterocyst development in *Anabaena*." Curr Opin Microbiol **6**(6): 557-63.

Golden, J. and K. S. Ramaswamy (1992). "Rearrangement of nif operons in cyanobacterial heterocysts. Abstr. no. 48, Abstr. 9th Intl. Congr. Nitrogen Fixation, Cancun, Mexico."

Golden, J. W. and D. R. Wiest (1991). "Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium Anabaena sp. strain PCC 7120. J. Bacteriol. 173:7098-7105."

Grula, J. W. (2005). "Evolution of Photosynthesis and Biospheric Oxygenation Contingent Upon Nitrogen Fixation?" J of Astrobiol **4**: 251-257.

Grunberg-Manago, M. (1999). "Messenger RNA Stability and its Role in Control of Gene Expression in Bacteria." Ann Rev of Genet **33**(1): 193-227.

Haselkorn, R. (1992). "Developmentally regulated gene rearrangements in prokaryotes." Annu. Rev. Genet. **26**: 113-130.

Haselkorn, R.. and S. J. Robinson (1983). "Organization and transcription of genes important in Anabaena heterocyst differentiation. Ann. Microbiol."

Haury, J. F. and H. Spiller (1981). "Fructose uptake and influence on growth of and nitrogen fixation by *Anabaena variabilis*." J Bacteriol **147**(1): 227-35.

Hawkesford, M. J.. Houchins, et al. (1983). "Reconstitution of photosynthetic electron transfer in cyanobacterial heterocyst membranes." FEBS Lett **159**(1-2): 262-266.

Henson, B. J., Pennington, et al. (2008). "Excision of the *nifD* element in the heterocystous cyanobacteria." Arch Microbiol **189**(4): 357-66.

Henson, B. J., Watson, et al. (2005). "Characterization of a 4 kb variant of the *nifD* element in Anabaena sp. strain ATCC 33047." Curr Microbiol **50**(3): 129-32.

Hernandez, J. Curatti, et al. (2008). "Metal trafficking for nitrogen fixation: NifQ donates molybdenum to NifEN/NifH for the biosynthesis of the nitrogenase FeMo-cofactor." Proc Nat Acad of Sci **105**(33): 11679-11684.

Hernandez, J. A., Igarashi, et al. (2007). "NifX and NifEN exchange NifB cofactor and the VK-cluster, a newly isolated intermediate of the iron-molybdenum cofactor biosynthetic pathway." Mol Microbiol **63**(1): 177-192.

Herrero, A., A. M. Muro-Pastor, et al. (2001). "Nitrogen control in cyanobacteria." J Bacteriol **183**(2): 411-425.

Hochman, A. and R. H. Burris (1981). "Effect of oxygen on acetylene reduction by photosynthetic bacteria." J Bacteriol **147**(2): 492-9.

Homer, M. J., D. R. Dean, et al. (1995). "Characterization of the Protein and Its Involvement in the Metallocluster Assembly and Maturation of Dinitrogenase from Azotobacter vinelandii." J Biol Chem **270**(42): 24745-24752.

Howard, J. B. and D. C. Rees (2006). "How many metals does it take to fix $N_2$? A mechanistic overview of biological nitrogen fixation." Proc Natl Acad of Sci U S A **103**(46): 17088-17093.

Hu, Y., M. C. Corbett, et al. (2006). "FeMo cofactor maturation on NifEN." Proc Natl Acad of Sci U S A **103**(46): 17119-17124.

Hu, Y., M. C. Corbett, et al. (2006). "Nitrogenase Fe protein: A molybdate/homocitrate insertase." Proc Natl Acad of Sci U S A **103**(46): 17125-17130.

Hu, Y., A. W. Fay, et al. (2005). "Identification of a nitrogenase FeMo cofactor precursor on NifEN complex." Proc Natl Acad Sci U S A **102**(9): 3236-41.

Hu, Y., J. M. Yoshizawa, et al. (2009). "Catalytic activities of NifEN: Implications for nitrogenase evolution and mechanism." Proc Nat Acad of Sci U S A 106(40): 16962-1696**6.**

Huang, X., Y. Dong, et al. (2004). "HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS." Proc Natl Acad Sci U S A.

Igarashi, R. Y. and L. C. Seefeldt (2003). "Nitrogen fixation: the mechanism of the Mo-dependent nitrogenase." Crit Rev Biochem Mol Biol 38(4): 351-84.

Igarashi, R. Y. and L. C. Seefeldt (2003). "Nitrogen Fixation: The Mechanism of the Mo-Dependent Nitrogenase." Rev Biochem and Mol Biol 38(4): 351-384.

Imperial, J., R. A. Ugalde, et al. (1984). "Role of the *nifQ* gene product in the incorporation of molybdenum into nitrogenase in *Klebsiella pneumoniae*." J. Bacteriol. 158(1): 187-194.

Iwasaki, H., S. B. Williams, et al. (2000). "A KaiC-interacting sensory histidine kinase, SasA, necessary to sustain robust circadian oscillation in cyanobacteria." Cell 101(2): 223-33.

Jackman, D. M. and M. E. Mulligan (1995). "Characterization of a nitrogen-fixation (*nif*) gene cluster from *Anabaena azollae* 1a shows that closely related cyanobacteria havehighly variable but structured intergenic regions." Microbiol 141(9): 2235-2244.

Jacobson, M. R., Cash, et al. (1989). "Biochemical and genetic analysis of the nifUSVWZM cluster from Azotobacter vinelandii." Mol Gen Genet 219(1-2): 49-57.

Jensen, B. (1990). "Fructose utilization by the cyanobacterium *Anabaena variabilis* studied using whole filaments and isolated heterocysts." Arch Microbiol **154**(1): 92-98.

Johnson, D. C., Dean, et al. (2005). "Structure Function and Formation of Biological Iron Sulfur Clusters." Ann Rev of Biochem 74(1): 247-281.

Joset, F., T. Buchou, et al. (1988). "Physiological and genetic analysis of the glucose-fructose permeation system in two *Synechocystis* species." Arch Microbiol 149(5): 417-421.

Kanehisa, M., M. Araki, et al. (2008). "KEGG for linking genomes to life and the environment." Nucl. Acids Res. 36(suppl-1): D480-484.

Kashyap, A. K. and G. Johar (1984). "Genetic control of ammonium transport in nitrogen fixing cyanobacterium Nostoc muscorum." Mol Gen Genet 197(3): 509-512.

Katoh, T. and K. Ohki (1975). "Loss of photosystem II induced by a nitrate deficiency in photoorganotrophically grown Anabaena variabilis." Plant Cell Physiol. 16(5): 815-828.

Khudyakov, I. Y. and J. W. Golden (2001). "Identification and inactivation of three group 2 sigma factor genes in *Anabaena* sp. strain PCC 7120." J. Bacteriol. 183(22): 6667-6675.

Khudyakov, I. Y. and J. W. Golden (2004). "Different functions of HetR, a master regulator of heterocyst differentiation in Anabaena sp. PCC 7120, can be separated by mutation." Proc Natl Acad Sci U S A 101(45): 16040-**5.**

Kkarecha, P., Kasting, J., Seifiert, J. (2005). "A coupled atmosphere-ecosystem model of the early archean earth." Geobiol 3: 57-76.

Komarek J. (2005). "The modern classification of Cyanoprokaryotes (Cyanobacteria),." Ocean. Hydrob. Studies 34(Supl.3): 5-17.

Kondo, T., Ishiura M. (1994). "Circadian rhythms of cyanobacteria: monitoring the biological clocks of individual colonies by bioluminescence. J Bacteriol 176: 1881-1885."

Kondo, T., Strayer C. A. Kulkarni R. D. Taylor W. Ishiura M. Golden S. S. Johnson C. H. (1993). "Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc Natl Acad Sci USA 90: 5672-5676."

Kumar, A. P. and H. N. Singh (1985). "Nitrate reductase activity in isolated heterocysts of the cyanobacterium Nostoc muscorum. FEBS Lett. 179:125-128."

Lambein, F. a. W., C. P., (1973). "Structural studies on the glycolipids from the envelope of the heterocyst of Anabaena cylindrica." Biochem **12**: 791-798.

Lang, N. J., J. M. Krupp, et al. (1987). "Morphological and ultrastructural changes in vegetative cells and heterocysts of *Anabaena variabilis* grown with fructose." J Bacteriol **169**(2): 920-3.

Laurent, S., H. Chen, et al. (2005). "Nonmetabolizable analogue of 2-oxoglutarate elicits heterocyst differentiation under repressive conditions in *Anabaena* sp. PCC 7120." Proc Natl Acad Sci U S A **102**(28): 9907-12.

Lavrrar, J. L. and M. A. McIntosh (2003). "Architecture of a Fur Binding Site: a Comparative Analysis." J. Bacteriol. 185(7): 2194-2202.

Leigh, G. J. (1995). "The mechanism of dinitrogen reduction by molybdenum nitrogenases." Eur J Biochem 229(1): 14-20.

Leigh, G. J. (2004). Nitrogen Fixation at the Millennium. Amsterdam, New York, preface, Elsevier Science BV.

Lex, M. and N. G. Carr (1974). "The metabolism of glucose by heterocysts and vegetative cells of Anabaena cylindrica." Arch Microbiol 101(1): 161-167.

Li, Y. and S. Altman (2003). "A specific endoribonuclease, RNase P, affects gene expression of polycistronic operon mRNAs." Proc Natl Acad of Sci U S A 100(23): 13213-13218**.**

López-Gomollón, S., J. A. Hernández, et al. (2007). "Cross-talk Between Iron and Nitrogen Regulatory Networks in Anabaena (Nostoc) sp. PCC 7120: Identification of Overlapping Genes in FurA and NtcA Regulons." J Mol Biol 374(1): 267-281.

Lyons, E. M. and T. Thiel (1995). "Characterization of *nifB*, *nifS*, and *nifU* genes in the cyanobacterium *Anabaena variabilis*: NifB is required for the vanadium-dependent nitrogenase." J Bacteriol 177(6): 1570-5.

Mannan, R. M. and H. B. Pakrasi (1993). "Dark heterotrophic growth conditions result in an increase in the content of photosystem II units in the filamentous cyanobacterium Anabaena variabilis ATCC 29413." Plant Physiol 103(3): 971-7.

Mariscal, V., A. Herrero, et al. (2007). "Continuous periplasm in a filamentous, heterocyst-forming cyanobacterium." Mol Microbiol 65(4): 1139-1145.

Markowitz, V. M., F. Korzeniewski, et al. (2006). "The integrated microbial genomes (IMG) system." Nucl. Acids Res. 34(suppl_1): D344-348

Martin-Figueroa, E., F. Navarro, et al. (2000). "The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of glsF gene from the cyanobacterium *Anabaena* sp. PCC 7120." FEBS Lett 476(3): 282-6.

Masepohl, B. S., Y. Drepper, A. Muller, A. Klipp, W (2002). Alternative nitrogenases. Nitrogen fixation at the millenium. J. Leigh.

Meeks, J. C. (2005). The genome of the filamentous cyanobacterium *Nostoc punctiforme*. Genomes and Genomics of Nitrogen-Fixing Organisms. R. Palacios and W. E. Newton. Dordrecht, Netherlnda, Springer: 27-70.

Meeks, J. C. (2006). Molecular mechanisms in the nitrogen fixing *Nostoc*-bryophyte symbiosis. Molecular Basis of Symbiosis. J. Overmann. Dordrecht, Springer: 165-196.

Meeks, J. C. and J. Elhai (2002). "Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states." Microbiol Mol Biol Rev 66(1): 94-121.

Meeks, J. C., J. Elhai, et al. (2002). "An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium." Photosynth Res 70: 85-106.

Meeks, J. C., C. M. Joseph, et al. (1988). "Organization of the nif genes in cyanobacteria in symbiotic association with *Azolla* and *Anthoceros*." Arch Microbiol 150(1): 61-71.

Meeks, J. and A. Galonsky (1977). "The pathways of assimilation of $^{13}NH_4$+ by the cyanobacterium, *Anabaena cylindrica*. J. Biol. Chem. 252:7894-7900."

Merida, A., E. Flores, et al. (1992). "Regulation of Anabaena sp. strain PCC 7120 glutamine synthetase activity in a *Synechocystis* sp. strain PCC 6803 derivative

strain bearing the Anabaena glnA gene and a mutated host glnA gene." J Bacteriol **174**(2): 650-4.

Miller, J. (1992). "A Short Course in Bacterial Genetics (Cold Spring Harbor Lab. Press, Plainview, NY),." 72-74.

Milligan, A. J., I. Berman-Frank, et al. (2007). "Light-dependent oxygen consumption in nitrogen-fixing cyanobacteria plays a key role in nitrogenase protection." J Phyco **43**(5): 845-852.

Modak, J. M. (2002). "Haber Process for Ammonia Synthesis." Resonance **7**: 69-77.

Montoya, J. P. (2004). "High rates of $N_2$ fixation by unicellular diazatrophs in the oligotrophic pacific ocean." Nature(430): 1027-1041.

Mori, S., A. Castoreno, et al. (2003). "Nitrogen status modulates the expression of RNA-binding proteins in cyanobacteria." FEMS Microbiol Lett **227**(2): 203-210.

Mulligan, M. E. and R. Haselkorn (1989). "Nitrogen fixation (*nif*) genes of the cyanobacterium Anabaena species strain PCC 7120. The *nifB-fdxN-nifS-nifU* operon." J Biol Chem **264**(32): 19200-7.

Mulligan, M. E., D. M. Jackman, et al. (1994). "Heterocyst-forming Filamentous Cyanobacteria Encode Proteins that Resemble Eukaryotic RNA-binding Proteins of the RNP Family." J of Mol Biol **235**(3): 1162-1170.

Muro-Pastor, A., E. Flores, et al. (2009). "NtcA-Regulated Heterocyst Differentiation Genes *hetC* and *devB* from *Anabaena* sp. Strain PCC 7120 Exhibit a Similar Tandem Promoter Arrangement." J. Bacteriol. **191**(18): 5765-5774.(NCBI), G. P. R.

Newbury, S. F., Smith, et al. (1987). "Differential mRNA stability controls relative gene expression within a polycistronic operon." Cell **51**(6): 1131-1143.

Nicolaisen, K., A. Hahn, et al. (2009). "The cell wall in heterocyst formation by Anabaena sp. PCC 7120." J Basic Microbiol **49**(1): 5-24.

Nicolaisen, K., V. Mariscal, et al. (2009). "The outer membrane of a heterocyst-forming cyanobacterium is a permeability barrier for uptake of metabolites that are exchanged between cells." Mol Microbiol **74**(1): 58-70.

Nieva, M. and E. F. Valiente (1996). "Inorganic Carbon Transport and Fixation in Cells of *Anabaena variabilis* Adapted to Mixotrophic Conditions." Plant Cell Physiol. **37**(1): 1-7.

Nyhus, K. J., T. Thiel, et al. (1993). "Targeted interruption of the *psaA* and *psaB* genes encoding the reaction-centre proteins of photosystem I in the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413." Mol Microbiol **9**: 979-988.

Orozco, C., D. Risser, et al. (2006). "Epistasis analysis of four genes from Anabaena sp. strain PCC 7120 suggests a connection between PatA and PatS in heterocyst pattern formation." J Bacteriol **188**(5): 1808-16.

Osanai, T., M. Azuma, et al. (2007). "Sugar catabolism regulated by light- and nitrogen-status in the cyanobacterium *Synechocystis* sp. PCC 6803." Photochem Photobiol Sci **6**(5): 508-14.

Osanai, T., S. Imamura, et al. (2006). "Nitrogen induction of sugar catabolic gene expression in *Synechocystis* sp. PCC 6803." DNA Res **13**(5): 185-95.

Osanai, T., Y. Kanesaki, et al. (2005). "Positive regulation of sugar catabolic pathways in the cyanobacterium *Synechocystis* sp. PCC 6803 by the group 2 sigma factor *sigE*." J Biol Chem **280**(35): 30653-9.

Ownby, J. D., M. Shannahan, et al. (1979). "Protein Synthesis and Degradation in Anabaena During Nitrogen Starvation J Gen Microbiol **110**(2): 255-261.

Peterson, J. B. (1989). "Respiratory differences associated with culture aeration in Azotobacter vinelandii." Canadian J Microbiol.

Pils, D., C. Wilken, et al. (2004). "Respiratory terminal oxidases in the facultative chemoheterotrophic and dinitrogen fixing cyanobacterium *Anabaena variabilis* strain ATCC 29413: characterization of the *cox2* locus." Biochim Biophys Acta **1659**(1): 32-45.

Poole, R. K. and S. Hill (1997). "Respiratory Protection of Nitrogenase Activity in Azotobacter vinelandiiâ€"Roles of the Terminal Oxidases." Bioscience Reports **17**(3): 303-317.

Porchia, A. C. and G. L. Salerno (1996). "Sucrose biosynthesis in a prokaryotic organism: presence of two sucrose-phosphate synthases in *Anabaena* with remarkable differences compared with the plant enzymes." Proc. Natl. Acad. Sci. USA **93**: 13600-13604.

Pratte, B. S., K. Eplin, et al. (2006). "Cross-functionality of nitrogenase components NifH1 and VnfH in Anabaena variabilis." J Bacteriol **188**(16): 5806-11.

Pratte, B. S. and T. Thiel (2006). "High-affinity vanadate transport system in the cyanobacterium Anabaena variabilis ATCC 29413." J Bacteriol **188**(2): 464-8.

R L Smith, D. K., X K Zhang, F R Tabita, and C Van Baalen (1985). "$H_2$, $N_2$, and $O_2$ metabolism by isolated heterocysts from *Anabaena* sp. strain CA." J Bacteriol. **162**(2): 565-570.

Ramasubramanian, T. S., Wei, et al. (1994). "Two *Anabaena* sp. strain PCC 7120 DNA-binding factors interact with vegetative cell- and heterocyst-specific genes." J Bacteriol **176**: 1214-1223.

Ramasubramanian, T. S., Wei, et al. (1996). "Transcription of the *Anabaena* sp. strain PCC 7120 *ntcA* gene: multiple transcripts and NtcA binding." J. Bacteriol. **178**: 922-926.

Ramaswamy, K. S., Carrasco, et al. (1997). "Cell-type specificity of the Anabaena fdxN-element rearrangement requires *xisH* and *xisI*." Mol Microbiol **23**(6): 1241-9.

Ramirez, M. E., Hebbar, et al. (2005). "*Anabaena* sp. strain PCC 7120 gene *devH* is required for synthesis of the heterocyst glycolipid layer." J Bacteriol **187**(7): 2326-31.

Rangaraj, P. and P. W. Ludden (2002). "Accumulation of 99Mo-containing Iron-Molybdenum Cofactor Precursors of Nitrogenase on NifNE, NifH, and NifX ofAzotobacter vinelandii." J Biol Chem **277**(42): 40106-40111.

Reddy, P. M., H. Spiller, et al. (1996). "Photodissimilation of fructose to $H_2$ and $CO_2$ by a dinitrogen-fixing cyanobacterium, *Anabaena variabilis*." Appl Environ Microbiol **62**(4): 1220-1226.

Rippka, R., J. Deruelles, et al. (1979). "Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria ." J Gen Microbiol 111(1): 1-61.

Rozen, A., H. Arad, et al. (1986). "Fructose supports glycogen accumulation, heterocysts differentiation, $N_2$ fixation and growth of the isolated cyanobiont *Anabaena azollae*." Arch Microbiol 145(2): 187-190.

Rozen, A., M. Schönfeld, et al. (1988). "Fructose-enhanced development and growth of the $N_2$-fixing cyanobiont *Anabaena azollae*." Z. Naturforsch. 43c: 408-412.

Rubio, L. M. and P. W. Ludden (2005). "Maturation of nitrogenase: a biochemical puzzle." J Bacteriol 187(2): 405-14.

Rubio, L. M. and P. W. Ludden (2008). "Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase." Ann Rev of Microbiol 62(1): 93-111.

Rubio, L. M., P. Rangaraj, et al. (2002). Cloning and Mutational Analysis of the Î³ Gene from Azotobacter vinelandii Defines a New Family of Proteins Capable of Metallocluster Binding and Protein Stabilization J Biol Chem 277(16): 14299-14305.

Rubio, L. M., S. W. Singer, et al. (2004). Purification and characterization of NafY (apodinitrogenase gamma subunit) from *Azotobacter vinelandii*. J Biol Chem 279(19): 19739-46.

Sato, N. and A. Wada. (1996). Disruption analysis of the gene for a cold-regulated RNA-binding protein, rbpA1, in *Anabaena*: cold-induced initiation of the heterocyst differentiation pathway. Plant Cell Physiol. 37: 1150-1160.

Sawers, R. G. (2006). Differential turnover of the multiple processed transcripts of the *Escherichia coli focA-pflB* operon. Microbiol 152(8): 2197-2205.

Schibler, U. and F. Sierra (1987). Alternative Promoters in Developmental Gene Expression. Ann Rev Genet 21(1): 237-257.

Schilling, N. and K. Ehrnsperger (1985). "Cellular differentiation of sucrose metabolism in *Anabaena variabilis*. Z. Nature 40c:776-779."

Schmetterer, G. and E. Flores (1988). Uptake of fructose by the cyanobacterium *Nostoc* sp. ATCC 29150." Biochim Biophys Acta. **942**: 33–37.

Schmetterer, G., A. Valladares, et al. (2001). The *coxBAC* operon encodes a cytochrome c oxidase required for heterotrophic growth in the cyanobacterium Anab*a*ena *variabilis* strain ATCC 29413. J Bacteriol **183**(21): 6429-34.

Shah VK, A. J., Spangler NJ, Ludden PW. (1994). In vitro synthesis of the iron-molybdenum cofactor of nitrogenase. Purification and characterization of NifB cofactor, the product of NifB protein. J Biol Chem **269**(2): 1154-8.

Shah, V. K., Davis, et al. (1975). Nitrogenase. VI. Acetylene reduction assay: Dependence of nitrogen fixation estimates on component ratio and acetylene concentration. Biochim Biophys Acta **384**(2): 353-9.

Shi, D.-J. and D. Hall (1988). The Azolla-*Anabaena* association: Historical perspective, symbiosis and energy metabolism. Botanical Rev **54**(4): 353-386.

Shi, Y., W. Zhao, et al. (2006). Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NtcA in Anabaena sp. PCC 7120." Proc Natl Acad Sci U S A **103**(30): 11334-9.

Singh, A. K. and L. A. Sherman (2005). Pleiotropic effect of a histidine kinase on carbohydrate metabolism in *Synechocystis* sp. strain PCC 6803 and its requirement for heterotrophic growth. J Bacteriol **187**(7): 2368-76.

Smith, A. J. (1983). Modes of cyanobacterial carbon metabolism. The Biology of the Cyanobacteria. N. G. Carr and B. A. Whitton. Berkeley, Univeristy of California Press**:** 47-85.

Smith, B. E., F. Campbell, et al. Biochemistry of Nitrogenase and the Physiology of Related Metabolism [and Discussion]. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences **317**(1184): 131-146.

Spiller, H., G. Bookjans, et al. (1983). Regulation of hydrogenase activity in vegetative cells of *Anabaena variabilis*. J Bacteriol **155**(1): 129-37.

Stacey, G., C. Van Baalen, et al. (1979). Nitrogen and ammonia assimilation in the cyanobacteria: Regulation of glutamine synthetase. Arch Biochem and Biophys **194**(2): 457-467.

Stewart, W. D. (1978). Nitrogen-fixing cyanobacteria and their associations with eukaryotic plants. Endeavour **2**(4): 170-179.

Stülke, J. (2002). Control of transcription termination in bacteria by RNA-binding proteins that modulate RNA structures. Arch of Microbiol **177**(6): 433-440.

Suh, M.-H., L. Pulakat, et al. (2002). Functional expression of the FeMo-cofactor-specific biosynthetic genes *nifEN* as a NifE-N fusion protein synthesizing unit in Azotobacter vinelandii. Biochemical and Biophysical Research Communications **299**(2): 233-240.

Summers, M. L. and J. C. Meeks (1996). Transcriptional regulation of *zwf*, encoding glucose-6-phosphate dehydrogenase, from the cyanobacterium *Nostoc punctiforme* strain ATCC 29133 Mol Microbiol **22**(3): 473-480.

Summers, M. L., J. G. Wallis, et al. (1995). "Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in nitrogen fixation and dark growth of the cyanobacterium *Nostoc* sp. strain ATCC 29133." J Bacteriol **177**(21): 6184-94.

Suzuki, T., H. Yoshimura, et al. (2007). AnCrpA, a cAMP receptor protein, regulates nif-related gene expression in the cyanobacterium *Anabaena* sp. strain PCC 7120 grown with nitrate." FEBS Lett **581**(1): 21-8.

Tamagnini, P., E. Leitao, et al. (2005). "Uptake hydrogenase in cyanobacteria: novel input from non-heterocystous strains." Biochem Soc Trans **33**(Pt 1): 67-9.

Tel-Or, E., Huflejt, et al. (1986). "Hydroperoxide metabolism in cyanobacteria." Arch Biochem Biophys **246**(1): 396-402.

Tel-Or, E. and W. Stewart "Photosynthetic Components and Activities of Nitrogen-Fixing Isolated Heterocysts of *Anabaena cylindrica*." Proc Royal Society London. Series B, Biol Sci **198**(1130): 61-86.

Thiel, T. (1993). "Characterization of genes for an alternative nitrogenase in the cyanobacterium *Anabaena variabilis*." J Bacteriol **175**: 6276-6286.

Thiel, T. (1996). "Isolation and characterization of the *vnfEN* genes of the cyanobacterium *Anabaena variabilis*." J Bacteriol **178**: 4493-4499.

Thiel, T., E. M. Lyons, et al. (1997). "Characterization of genes for a second Mo-dependent nitrogenase in the cyanobacterium *Anabaena variabilis*." J Bacteriol **179**(16): 5222-5.

Thiel, T., E. M. Lyons, et al. (1995). "A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium." Proc Natl Acad Sci USA **92**: 9358-9362.

Thiel, T. and B. Pratte (2001). "Effect on heterocyst differentiation of nitrogen fixation in vegetative cells of the cyanobacterium *Anabaena variabilis* ATCC 29413." J Bacteriol **183**(1): 280-6.

Thiel, T., B. Pratte, et al. (2002). "Transport of molybdate in the cyanobacterium Anabaena variabilis ATCC 29413." Arch Microbiol **179**(1): 50-6.

Thiel, T. and C. P. Wolk (1987). "Conjugal transfer of plasmids to cyanobacteria." Meth Enzymol **153**: 232-43.

Thomas, J. (1970). "Absence of the Pigments of Photosystem II of Photosynthesis in Heterocysts of a Blue-Green Alga." **228**(5267): 181-183.

Torrecilla, I., F. Leganes, et al. (2004). "A calcium signal is involved in heterocyst differentiation in the cyanobacterium Anabaena sp. PCC7120." Microbiol **150**(Pt 11): 3731-9.

Triplett, E. W. (2000). Prokaryotic nitrogen fixation. A model system for analysis of a biological process. Garland science.

Tumer, N. E., S. J. Robinson, et al. (1983). "Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen." Nature **306**: 337-342.

Ungerer, J. L., B. S. Pratte, et al. (2008). "Regulation of fructose transport and its effect on fructose toxicity in Anabaena spp." J Bacteriol **190**(24): 8115-25.

Valiente, E. F., M. Nieva, et al. (1992). "Uptake and Utilization of Fructose by *Anabaena variabilis* ATCC 29413. Effect on Respiration and Photosynthesis." Plant Cell Physiol. **33**(3): 307-313.

Valladares, A., E. Flores, et al. (2008). "Transcription activation by NtcA and 2-oxoglutarate of three genes involved in heterocyst differentiation in the cyanobacterium Anabaena sp. strain PCC 7120." J Bacteriol **190**(18): 6126-33.

Valladares, A., A. M. Muro-Pastor, et al. (1999). "Constitutive and nitrogen-regulated promoters of the *petH* gene encoding ferredoxin:$NADP^+$ reductase in the heterocyst-forming cyanobacterium *Anabaena* sp." FEBS Lett **449**(2-3): 159-64.

Vazquez-Bermudez, M. F., A. Herrero, et al. (2002). "2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus glnA* promoter." FEBS Lett **512**(1-3): 71-4.

Vioque, A. (1992). "Analysis of the gene encoding the RNA subunit of ribonuclease P from cyanobacteria." Nucleic Acids Res. **20**: 6331-6337.

Walsby, A. E. "The Permeability of Heterocysts to the Gases Nitrogen and Oxygen." Proc Royal Society of London. Series B, Biol Sci **226**(1244): 345-366.

Weckesser, J. and U. J. Jürgens (1988). "Cell walls and external layers." Methods Enzymol. **167**: 173-188.

Willison, J. C., J. Pierrard, et al. (1993). "Sequence and transcript analysis of the nitrogenase structural gene operon (*nifHDK*) of Rhodobacter capsulatus: evidence for intramolecular processing of *nifHDK* mRNA." Gene **133**(1): 39-46.

Winkenbach, F. and C. P. Wolk (1973). "Activities of enzymes of the oxidative and the reductive pentose phosphate pathways in heterocysts of a blue-green alga." Plant Physiol. **52**: 480-482.

Wolk, C. P. (1968). "Movement of carbon from vegetative cells to heterocysts in Anabaena cylindrica." J Bacteriol **96**(6): 2138-43.

Wolk, C. P., A. Ernst, et al. (1995). Heterocyst Metabolism and Development. The Molecular Biology of Cyanobacteria**:** 769-823.

Wong, F. C. and J. C. Meeks (2002). "Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation." Microbiol. **148**(Pt 1): 315-23.

Wood, N. B. and R. Haselkorn (1979). Proteinase activity during heterocyst differentiation in nitrogen-fixing cyanobacteria. Limited Proteolysis in Microorganisms. G. H. Cohen and H. Holzer. Bethesda, MD, U.S. DHEW Publication No. (NIH) 79-1591**:** 159-166.

Wu, X., D. Liu, et al. (2004). "patS minigenes inhibit heterocyst development of *Anabaena* sp. strain PCC 7120." J Bacteriol **186**(19): 6422-9.

Yisong Guoa, S. J. G., Luis M. Rubio and Stephen P. Cramera " NifB-co, a Key FeMo-co Precursor, is a $Fe_6S_9$ Core with an Interstitial Light Atom." 1108.

Yoon, H. S. and J. W. Golden (2001). "PatS and products of nitrogen fixation control heterocyst pattern." J. Bacteriol. 183(8): 2605-2613.

Yuvaniyama, P., J. N. Agar, et al. (2000). "NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein." Proc Natl Acad Sci USA 97(2): 599-604.

Zahalak, M., B. Pratte, et al. (2004). "Molybdate transport and its effect on nitrogen utilization in the cyanobacterium *Anabaena variabilis* ATCC 29413." Mol Microbiol 51(2): 539-49.

Zehr, J. P., M. T. Mellon, et al. (1998). "New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of Nitrogenase (*nifH*) genes." Appl Environ Microbiol 64(9): 3444-50.

Zhang, C.-C., M.-C. Durand, et al. (1989). "Molecular and genetical analysis of the fructose-glucose transport system in the cyanobacterium *Synechocystis* PCC6803." Mol Microbiol3(9): 1221-1229.

Zhang, C. C., R. Jeanjean, et al. (1998). "Obligate phototrophy in cyanobacteria: more than a lack of sugar transport." FEMS Microbiol Lett 161(2): 285-92.

Zhang, C. C., S. Laurent, et al. (2006). "Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals." Mol Microbiol 59(2): 367-75.

Zhao, Y., S.-M. Bian, et al. (2006). "Diversity of Nitrogenase Systems in Diazotrophs." J Integrative Plant Biol **48**(7): 745-755.

Zhao, Y., Y. Shi, et al. (2005). "CcbP, a calcium-binding protein from *Anabaena* sp. PCC 7120, provides evidence that calcium ions regulate heterocyst differentiation." Proc Natl Acad Sci U S A **102**(16): 5744-8.

Zheng, L., V. L. Cash, et al. (1998). "Assembly of Iron-Sulfur Clusters 10.1074/jbc.273.21.13264." J Biol Chem 273(21): 13264-13272.

Zheng, L., R. White, et al. (1997). "Purification of the Azotobacter vinelandii nifV-encoded homocitrate synthase." J. Bacteriol. **179**(18): 5963-5966.

Zheng, L., R. H. White, et al. (2002). "Mechanism for the Desulfurization of L-Cysteine Catalyzed by the *nifS* Gene Product." Biochem **33**(15): 4714-4720.

Zheng, L., R. H. White, et al. (1993). "Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis." Proc Natl Acad Sci USA **90**(7): 2754-2758.

Zhou, J. X., J. Zhou, et al. (2008). "Characterization of two glutaminases from the filamentous cyanobacterium *Anabaena* sp. PCC 7120." FEMS Microbiol Letters **289**(2): 241-249.

Zhou, R., X. Wei, et al. (1998). "Evidence that HetR protein is an unusual serine-type protease." Proc Natl Acad Sci USA **95**: 4959-4963.

Zimmerman, W. J. H., B. Rosen, et al. (1989). "Enzymatic, lectin, and morphological characterization and classification of presumptive cyanobionts from Azolla Lam." New Phytologist **113**(4): 497-503.