Transposon Mutagenesis to Identify Trans-Acting Regulatory Factors of the *nif* Genes in *Anabaena* sp. Strain PCC 7120

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When grown in the absence of combined nitrogen, the photosynthetic cyanobacterium *Anabaena* sp. strain PCC 7120 fixes nitrogen inside terminally differentiated cells called heterocysts. Differentiation is a complex process involving genome rearrangements to yield functional *nifHDK* and *nifB-fdxN-nifSU* operons. The regulation of expression of the *nifHDK* operon has been studied extensively in other nitrogen-fixing bacteria, but *nifHDK* regulation in *Anabaena* is not yet understood. This study used transposon mutagenesis to identify possible trans-acting factors which regulate *nifHDK* transcription in *Anabaena* 7120. We analyzed mutants to identify colonies which produced normal looking heterocysts but could not fix nitrogen. Northern blot analysis followed to determine if the *nifH* promoter was active. DNA was isolated from mutants in which *nifH* expression was down or off and direct cloning was used to isolate the transposon. Other mutants in heterocyst formation were also subjected to direct cloning.

INTRODUCTION

While all organisms need some form of nitrogen to live, the ability to utilize atmospheric nitrogen is confined to a few prokaryotic groups. Bacterial nitrogen fixation is interesting because while industrial processes fix nitrogen for fertilizer under harsh conditions such as high pressure and high temperature, bacteria can fix nitrogen under relatively benign conditions in the cell. Researchers originally studied nitrogen fixation to determine how it was that a bacterial enzyme, nitrogenase, could catalyze a reaction that was so difficult to carry out otherwise. However, once scientists began to study nitrogenase, they noticed that the regulation of the enzyme was at least as interesting as the enzyme itself. Now,

many researchers study the regulation of nitrogen fixation in a variety of diazotrophs, such as *Klebsiella pneumoniae* and *Rhodobacter capsulatis*.

The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 is another organism capable of combining atmospheric nitrogen into a biologically useful form. *Anabaena* is particularly interesting because it is capable of both nitrogen fixation and carbon fixation, in the form of photosynthesis. This species needs very few supplemented nutrients to survive. When *Anabaena* filaments are grown on media that contains a combined form of nitrogen, all cells are vegetative, carrying out photosynthesis. When the wild-type strain is grown on media without a combined nitrogen source, about ten percent of the vegetative cells differentiate into heterocysts, specialized cells in which nitrogen fixation takes place. Heterocysts have a multilayered gas-impermeable cell wall, which prevents nitrogenase from being inactivated by oxygen. Development of heterocysts is a complex process involving at least two genome rearrangements that occur in the region of the *nif* genes. An 11-kilobase pair (kb) fragment excises out from within the *nifD* gene and a 55-kb fragment excises out from within *fdxN*. Both DNA excisions are site-specific recombination events involving specific recombinases (4). These rearrangements yield the intact *nifHDK* operon and the *nifB-fdxN-nifSU* operon.

The *nifHDK* operon codes for the main structural genes of nitrogenase; in *Anabaena*, its mechanism of regulation is not known but regulation of *nifHDK* in other nitrogen-fixing organisms has been described. In the enteric bacterium *Klebsiella pneumoniae*, for example, a special σ factor, called σ^{54}, is required for RNA polymerase to recognize the *nifH* promoter. The product of the *nifA* gene is also required for transcription of the *nifHDK* genes (1). Experiments have shown that NifA catalyzes the isomerization of RNA polymerase holoenzyme to form the open complex required for transcription (7).

Since the *nifH* promoter is only active in heterocysts, we assume that there is a specific regulatory factor that allows *nifH* to function only in heterocyst cells. Further, since the σ^{54}/*nifA* mechanism is highly conserved among other nitrogen-fixing organisms, we believe that *Anabaena* as well must have trans-acting factors regulating *nifHDK* activity and that these factors might show homology to σ^{54} and NifA from other organisms such as *K. pneumoniae*. However, because *Anabaena* is phylogenetically distant from other nitrogen-fixing organisms, and undergoes a developmental process related to nitrogen fixation that is not seen in other diazotrophs, it is possible that *Anabaena* may utilize a novel method of regulation of nitrogen fixation.

Researchers have attempted to clone out *nifA* and σ^{54} genes from *Anabaena* with no result. It is possible that even if *Anabaena* does have *nifA* and σ^{54}, the genes may be sufficiently divergent from those in other nitrogen-fixing organisms to make cloning by homology impossible. Our approach is a genetic rather than molecular approach, which allows for identification of novel regulatory factors by function rather than homology. A possible downfall of a genetic approach is that certain regulatory mechanisms could be difficult to identify by inactivating one part; for example, if there are two *nifA*-like genes, then inactivation of one might give no identifiable phenotype, as with *Rhodobacter capsulatis* and its duplicate *nifA* genes (6). However, such redundant genes are not common in prokaryotes.

To identify regulatory factors, we created mutants of *Anabaena* PCC 7120 that could not fix nitrogen in air and then characterized the specific mutations involved. Wolk et al (3) developed an efficient mutagenesis procedure using a Tn5-derived transposon which is a potent mutagen and allows for easy identification of genes via direct cloning. We employed a modified version of this transposon that has an added promoter reading outward, allowing an extra selection for mutants in which expression of a neighboring gene is turned on. Our initial selection was for mutants

that could not grow well on plates without combined nitrogen. We checked these Fox^- mutants under the microscope for the presence of heterocysts to ensure that the inability to fix nitrogen was not simply a result of a mutation somehow blocking heterocyst formation. From our pool of Fox^-Het^+ mutants we isolated mRNA and performed Northern analysis with a *nifH* DNA probe. Our Northern blot revealed several mutants in which *nifH* expression was either reduced or completely depleted. We attempted to characterize the mutations by direct cloning of the regions flanking the transposon. We also noted several other interesting mutants with varied heterocyst number, presence, or morphology; we attempted to clone the tagged gene in these mutants as well in order to characterize the mutations.

MATERIALS AND METHODS

Growth conditions. Wild-type *Anabaena* sp. strain PCC 7120 was grown at 30°C in the light on a rotatory shaker in 100 ml BG-11 supplemented with nitrate (N^+) in 200-ml Erlenmeyer flasks. Low light conditions for the conjugations were achieved by covering the box of plates with an opaque cover, in the lighted incubator.

For RNA isolations, mutant strains were grown in the presence of nitrogen and erythromycin at concentrations of 2-4 μg/ml in 50 ml BG-11(N^+) in 125-ml Erlenmeyer flasks. To induce the formation of heterocysts, cultures were washed twice with BG-11(N^-), resuspended in 50 ml BG-11(N^-) without antibiotics, and kept under growth conditions.

Transposon mutagenesis and selection of mutants. Transposon mutagenesis of wild-type *Anabaena* was achieved by transferring the transposon plasmid, pRL1087bPro (Fig. 1), from an *E. coli* conjugal donor into *Anabaena* by conjugation. The plasmid carries transposon Tn5-1087bPro and an RK2 origin of

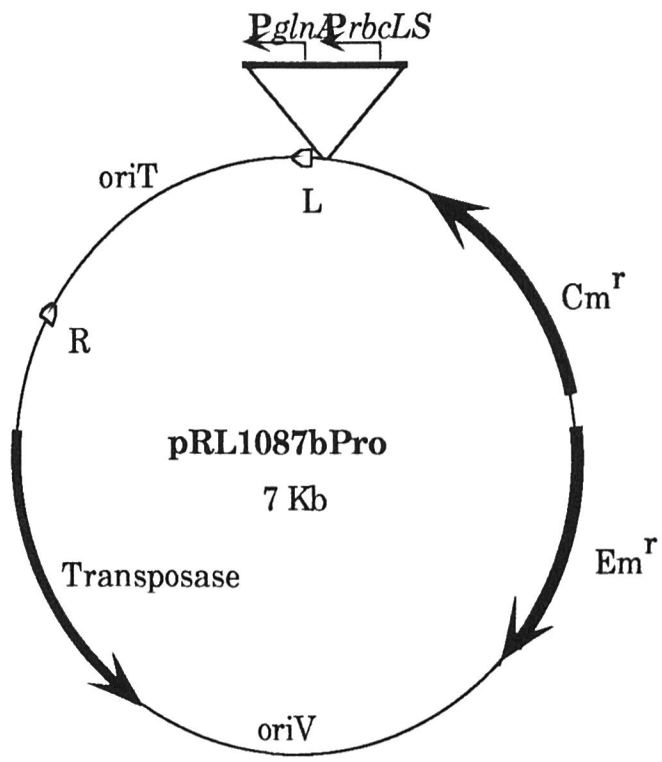

FIG. 1. Physical map of plasmid pRL1087bPro bearing transposon Tn5-1087bPro. L and R indicate the left and right ends of the transposon, respectively. Cm, gene conferring resistance to chloramphenicol; Em, gene conferring resistance to erythromycin.

transfer, but no cyanobacterial origin of replication. The transposon, Tn5-1087bPro, is a modified version of Tn5-1087b (3) with the addition of a strong promoter reading outward (Ramasubramanian, unpublished results). The transposon carries resistance markers for erythromycin and chloramphenicol, the *rbcL-glnA* promoter, and an *E. coli* origin of replication to allow for direct cloning.

The *E. coli* conjugal strain, containing an RP4 derivative, the pRL1045 helper plasmid (2), and pRL1087bPro, was grown up overnight in TB with antibiotics (ampicillin, chloramphenicol, kanamycin, and erythromycin). 1.5 ml of the *E. coli* culture was washed 3 times with TB to remove antibiotics, combined with *Anabaena* from 40 ml of a 6-8 day old culture, and centrifuged at 4000 rpm for 4 minutes. After removing supernatant, 2 ml of 95% BG-11(N^+) + 5% LB was added and conjugations were incubated 24 hours at 30°C in the light. The conjugations were then plated on 20 BG-11(N^+) plates with 6 μg/ml erythromycin (Em) and incubated overnight at

low light. The plates were then incubated at normal light intensity for 10-14 days. This procedure produced 200-500 Em-resistant colonies per plate.

RNA isolation and Northern analysis. Selected mutants were grown in liquid culture in the presence of nitrogen and erythromycin to mid-log phase and then induced to form heterocysts. When mature heterocysts were clearly visible under the microscope, usually at 28-40 hours, cultures were harvested by centrifuging at 4°C, removing supernatant, and freezing. Total RNA was isolated by hot phenol extraction after disrupting cells by treatment with 1% SDS at 65°C. RNA was precipitated and resuspended in DEPC-treated water.

RNA samples were denatured at 65°C in the presence of formamide and formaldehyde and run on formaldehyde gels. RNA was transferred from the gels to a charged nylon membrane (Magna Charge from MSI, Westboro, MA) by pressure blotting with the Posiblot system from Stratagene (La Jolla, CA). The blots were hybridized with an An154.3 probe which includes the *nifH* gene and upstream sequences. The probe was labeled with [α-^{32}P]dCTP with a random primer labeling kit. Hybridizations were carried out in 50% formamide, 5X SSPE, and 1% SDS at 55°C. After hybridizing overnight, the membranes were washed with 0.5X SSPE and 0.1% SDS at 65°C for 20 minutes, 3 times. The signals were analyzed with a Fujix phosphoimager after exposing overnight on imaging plates.

DNA isolation and direct cloning. Chromosomal DNA was isolated from mutants by treatment with STET containing 1 mg/ml lysozyme, vortexing for 2 minutes, and incubating at 37°C for 10 minutes. Samples were treated with 1% CTAB in 0.7M NaCl, incubated 10 minutes at 65°C and extracted with phenol/chloroform. After precipitating and resuspending in TE, the DNA was digested with *Mlu*I (GIBCO BRL, Gaithersburg, MD), which cuts frequently within the chromosome but not within the transposon. Digested DNA was ligated in dilute

conditions to promote self-ligation and electroporated into *E. coli*. Transformants were selected with chloramphenicol.

Southern analysis. Insertion of the pRL1087bPro transposon in the mutants was confirmed by Southern analysis of DNA isolated from mutants and digested with *Hin*dIII. A probe containing the transposon was labeled with [α-^{32}P]dCTP using a random primer labeling kit.

RESULTS

Fox$^-$ phenotype. After generation of our mutant library, we began screening for colonies that were incapable of carrying out nitrogen fixation. Exconjugant colonies containing the transposon were transferred from plates with nitrogen to plates without nitrogen. Colonies that cannot fix nitrogen in air (Fox$^-$) grow normally at first on N$^-$ plates due to residual nitrogen compounds in the media. These colonies later begin to turn yellow and die. We found that roughly 10% of colonies picked to media without nitrogen exhibited this phenotype (Table 1). Fox$^-$ colonies were restreaked to media with nitrogen for preservation.

Fox$^-$ Het$^+$ phenotype. Since the goal of our experiment was to identify mutants that could not fix nitrogen due to lack of expression of *nifHDK*, we next eliminated mutants that were not fixing nitrogen due to the absence or abnormality of heterocysts. Fox$^-$ colonies were checked under the microscope for the presence of mature heterocysts. Out of 328 Fox$^-$ colonies, 96 were found to be Het$^+$, having near wild-type percentages of normal-looking heterocysts.

Fox$^-$Het$^+$NifH$^-$ phenotype. Mutants that were not fixing nitrogen but had normal-looking heterocysts were then checked for *nifH* expression. Northern blot analysis showed that very few mutants lacked *nifH* message altogether (FIG. 2). Rather, 11 mutants showed reduced *nifH* message (NifH^{down}) while only 2 mutants

TABLE 1. Isolation of transposon-generated mutants

Isolation step or phenotype	Number of colonies	Colony name
Tn-generated colonies	> 12,000	
transferred to BG-11_0	4000	
Fox^-	328	
Fox^- Het^+	96	
putative Fox^- Het^+ $NifH^-$	22	(out of 85 analyzed)
Fox^- Het^+ $NifH^{down}$	17	(*nifH* message <20% of control)
Fox^- Het^+ $NifH^{zero}$	5	(*nifH* message <1% of control)
Other Phenotypes		
Fox^- Het^-	158	(preliminary observations)
Fox^- Het^+(less frequent)	52	
Fox^- Hen^-	5	II-1, VI-2, XV-7, XXVII-5, XXVIII-7
Fox^- Het^+(terminal hets)	4	I-5, I-12, XXII-4, XXV-1
Fox^- Het^+(prohets)	3	I-1, I-2, XXVIII-4
Fox^- Het^+(no polar body)	2	VI-3, XI-9
Fox^- Het^c(hets on $NaNO_3$)	2	I-4, XXII-5

showed no *nifH* message at all. The next step of the mutant screen was to clone the transposon and flanking DNA out of the *Anabaena* chromosome and sequence the interrupted gene. We isolated genomic DNA from each of the $NifH^-$ and $NifH^{down}$ mutants and attempted to clone the transposon out of the chromosome by direct cloning. So far we have obtained transformants that have the transposon but no flanking DNA.

Other mutant phenotypes. Many other mutant phenotypes were discovered among the Fox^- mutants (Table 1). Of the 328 Fox^- mutants, 52 had less than wild-type percentages of heterocysts, while 158 mutants had no heterocysts. Some of the mutants had abnormal hets. For example, 5 of the mutants had abnormal envelopes, 2 of the mutants had no polar bodies, and 3 of the mutants could only form proheterocysts, not mature heterocysts. Four of the mutants formed only terminal hets, while two mutants formed hets constitutively.

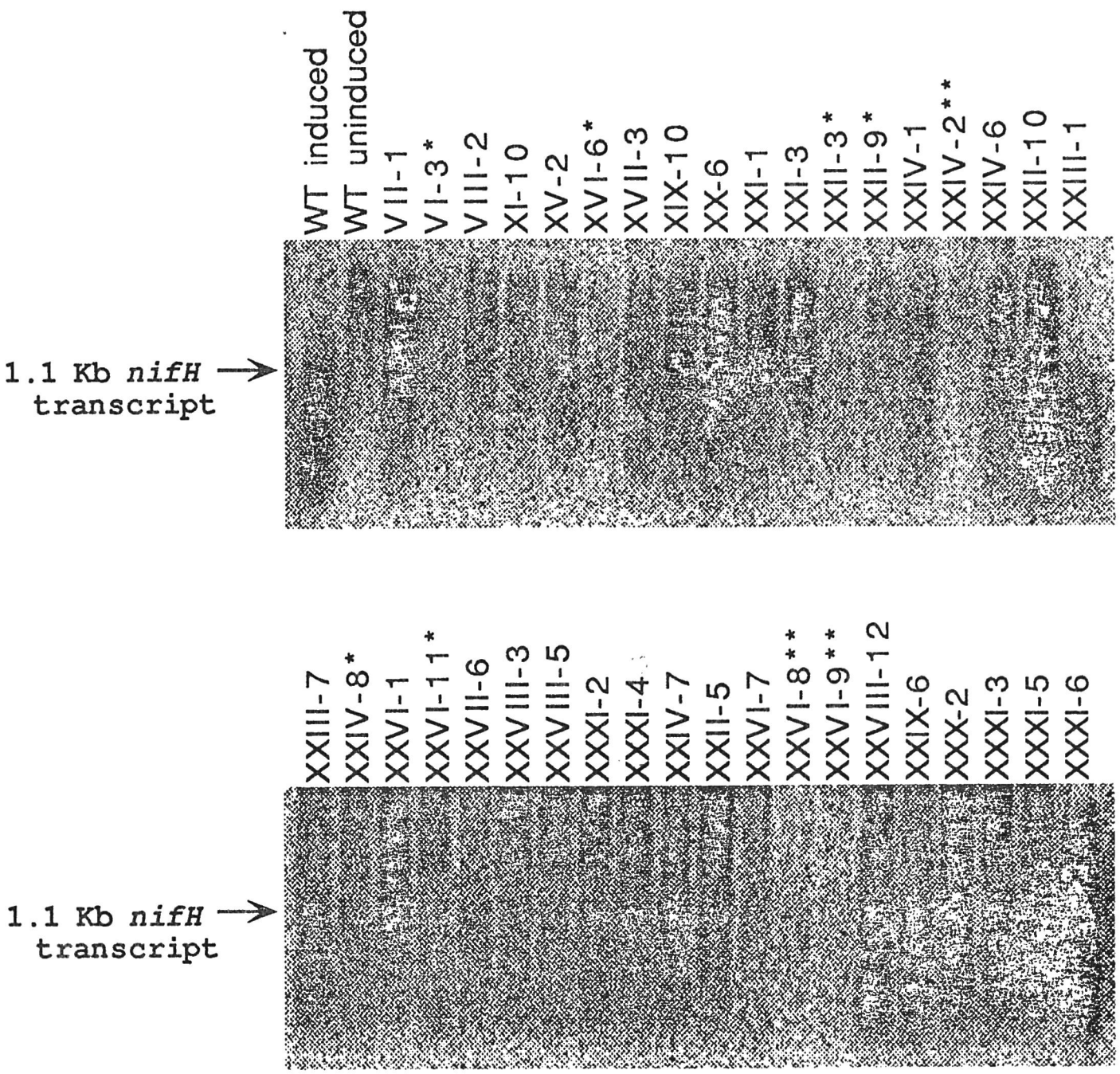

FIG. 2. Transcription of the *nifHDK* operon in selected mutants. Northern blots of RNA samples from wild-type (WT) induced, WT uninduced, and Fox$^-$Het$^+$ mutants induced. Samples were probed with a labeled An154.3 probe including the *nifH* gene and upstream sequences.

* *nifH* expression down, <20% of control

** *nifH* expression off, <1% of control

DISCUSSION

To find regulatory factors affecting *nifHDK* expression in *Anabaena* we used transposon mutagenesis to create a mutant library and then screened for the mutants that we wanted. We successfully generated mutants in which *nifH* expression was affected, as well as several other mutants that will be characterized later. Now that we have our desired mutants, the next step is to clone out the transposon and flanking regions, use the flanking DNA to probe an *Anabaena* library for an uninterrupted copy of the gene, and determine its DNA sequence. The regulatory gene(s) may have homology to other *nif* regulators from other systems, or we may identify novel regulatory factors in *Anabaena*.

Since we are having difficulty cloning the transposon along with flanking DNA, we have been considering new strategies for obtaining clones. We think that our problem might be that the enzyme we have been using to break up the *Anabaena* chromosome is not producing fragments of a size useful for the cloning. However, there are many other ways to break up large pieces of DNA. For example, we could physically shear the DNA by passing it through a syringe needle. Another method for physically breaking up the chromosome is by sonication. These methods could allow us to generate fragments of a more useful size.

We obtained a few mutants with a $NifH^-$ phenotype. Our initial interpretation of this phenotype was that a gene for a positive regulatory factor was disrupted by the transposon. However, it is possible that the *nifH* mRNA was unstable and degraded before we harvested the cells. Normally, *nifH* transcription peaks about 24 hours after induction (5), but since we were growing mutants, we could not be sure how growth rate would be affected by the mutations. With many different mutants, it was difficult to choose a harvest time that would permit maximum *nifH* transcript recovery for all of them. Another consideration is that the mutations could change

the timing of expression of *nifH* so that certain mutants could be peaking in *nifH* expression before or after we harvested the cells. The final stage of the experiment, cloning and sequencing the region flanking the transposon, will provide more conclusive information.

The NifH$^-$ mutants may not even be as interesting as the NifH^{down} mutants we identified, since the NifH$^-$ mutants may simply have the transposon inserted into the *nifH* promoter or the coding region. A missing trans-acting factor may not result in a completely non-expressing phenotype; a gene that is missing an activator may be expressed at a low level. Therefore, the NifH^{down} mutants are likely to have an intact *nifH* gene with a missing activator.

We are also interested in characterizing the mutations causing the other phenotypes we described. Many mutants exhibited a Het$^-$ phenotype. Perhaps a gene for sensing or responding to the presence of nitrate has been lost. These mutants would be unable to sense nitrogen-limiting conditions, so that heterocyst development is not stimulated. The Het$^-$ phenotype and other phenotypes, such as the mutants that could only form proheterocysts, and those in which the polar bodies were absent, could be explained by the absence of an early sigma factor that is required for a developmentally regulated process. Another possibility is that a catabolic enzyme was disrupted, which slowed development. While some cultures showed fully mature heterocysts, cultures in which this catabolic enzyme was nonfunctional would still be in an earlier developmental stage.

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