Final Report

TITLE: Regulation of Development and Nitrogen Fixation in *Anabaena*

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Summary

The specific aims for this project focused on the critical regulator HetR and on the transcriptional regulation of *nif* genes. The discovery that HetR has *in vitro* DNA-binding activity, which is inhibited by synthetic PatS-5 pentapeptide (5), resulted in a change in our research priorities. These new data made it very likely that HetR itself is the long-sought-after transcription factor that acts as the master regulator leading to heterocyst differentiation. Although we are continued work on HetR modification, our proposed experiments to identify HetR-interacting proteins were not actively pursued. We also shifted our focus to research goals more directly related to the DOE mission and EB research activities. Therefore, we devoted our efforts to the purification of *Anabaena* RNAP holoenzyme to elucidate the mechanisms that regulate heterocyst-specific genes, focusing on the *nif* genes. Our work on HetR and *nif* genes is presented below. Results regarding uptake hydrogenase genes and the novel gene *asr1734*, which were partially supported by DOE funding, are also briefly presented.

Our accomplishments supported by the DOE are summarized here.

- Identification and analysis of the *hetR*(R223W) mutant was completed and published.
- HetR protein was purified and anti-HetR antiserum was obtained and was used for experiments to track HetR modification and localization.
- Several yeast two-hybrid constructions were made to test for interactions among: HetR, PatS, PatA, HetF, and Asr1734.
- Three *nifH* promoter fusions to *gfp* were constructed, conjugated into *Anabaena*, and characterized.
- We constructed a plasmid that contains the *hglE* promoter fused to *gfp*. Heterocyst-specific expression of P_{hglE}-*gfp* was observed in a reporter strain.
- We constructed His-tagged versions of the *Anabaena* RNAP beta (*alr1594*) and beta' (*alr1596*) subunits in shuttle vectors for experiments with RNAP holoenzyme isolated from different samples and for *in vitro* transcription assays.

DOE funding partially or indirectly supported the following projects.

- Identification and analysis of the gene *asr1734*, which blocks development of a *hetR*(R223W) strain.
- The expression of sigma factor genes has been followed by using *gfp* reporter fusions; sigma factors SigC, SigF, and SigG are upregulated in differentiating cells.
- Three oligopeptide permease (*opp*) genes were knocked out to test a hypothesis that they are required for cell-cell signaling and heterocyst pattern formation.

Publications resulting from this project

Lee, M. H., M. Scherer, S. Rigali, and J. W. Golden. 2003. PlmA, a new member of the GntR family, has plasmid maintenance functions in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **185:**4315-4325. http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=12867439

Golden, J. W., and H.-S Yoon. 2003. Heterocyst development in *Anabaena*. Curr. Opin. Microbiol. **6**: 557-563. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14662350

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. USA **101:**16040-5. http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=15520378

Wu, X., D. W. Lee, R. A. Mella, and J. W. Golden. 2007. The *Anabaena* sp. strain PCC 7120 *asr1734* gene encodes a negative regulator of heterocyst development. Mol. Microbiol. **64**:782-794. http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05698.x

Aldea, M. R., R. A. Mella, and J. W. Golden, 2007. Sigma factor genes *sigC*, *sigF*, and *sigL* are upregulated in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **189**: 8392-8396. http://jb.asm.org/cgi/content/abstract/189/22/8392

Aldea, M. R., K. Kumar, and J. W. Golden, 2008. Heterocyst development and pattern formation, p. 75-90, *In* S. C. Winans and B. L. Bassler (eds.), Chemical Communication Among Microbes. ASM Press, Washington, D.C.

Research progress

The following sections summarize our research supported by the DOE. Recent data showing that HetR is potentially a transcription factor have caused us to shift our research priorities to the regulation of heterocyst-specific late genes including the *nifH*, *fdxH*, and *hupS* genes, and also genes important for heterocyst morphogenesis such as the heterocyst glycolipid (*hgl*) genes, which are expressed around the time of commitment to differentiate. We have initiated work that will allow us to isolate RNAP holoenzyme from *Anabaena* for analysis of the associated sigma factors and for *in vitro* transcription assays.

HetR

HetR, long recognized as a key player in heterocyst development, is now clearly established as the central regulator because of the discovery of its DNA-binding activity. Our characterization of a *hetR*(R223W) allele, which is insensitive to inhibition by the patterning signals PatS and HetN, was

published in PNAS (7). We became aware of the DNA-binding activity of HetR near the beginning of the current funding period, which caused a change in the major goal of our previously proposed research. We shifted our HetR-related research to investigate the potential regulation of HetR activity by covalent modification, and decided that we must put much more emphasis on the biochemistry of transcriptional regulation during heterocyst differentiation.

A search for mutants of *Anabaena* that can overcome heterocyst suppression caused by overexpression of the *patS* gene yielded a bypass mutant, S2-45, that produces a defective pattern (**Pat** phenotype) of irregularly spaced single and multiple contiguous heterocysts (**Mch** phenotype) in combined nitrogen-free medium. Analysis of the S2-45 mutant revealed an Arg to Trp substitution (R223W) in HetR, and reconstruction in the wild-type (WT) background showed that this mutation is responsible for the Mch phenotype and for resistance to overexpressed *patS* or *hetN*, another negative regulator of differentiation. Ectopic overexpression of the *hetR*(R223W) allele in the *hetR*(R223W) background resulted in a conditionally lethal phenotype (nearly all cells differentiate). Analysis of the heterocyst pattern in the *hetR*(R223W) mutant revealed that heterocysts differentiate essentially randomly along filaments, indicating that this mutation results in an active protein that is insensitive to the major inhibitory signals that govern heterocyst pattern formation. These data provide genetic evidence that, apart from being an activator of differentiation, HetR plays a central role in the signaling pathway that controls the heterocyst pattern.

HetR Modification

Native HetR protein from *Anabaena* shows multiple bands on gels with different isoelectric points that differ from recombinant HetR expressed in *E. coli* (9). The relative levels of these different forms shift after nitrogen step-down, indicating that HetR undergoes posttranslational modifications during development. These modifications may serve to regulate HetR activity by changing its interaction with other proteins, modifying its sensitivity to inhibitors such as PatS, or regulating its DNA-binding or auto-protease activity. It is also possible that different modified forms of HetR are present in different cell types. To test these possibilities, it first is necessary to characterize the type, site, and stability of the modifications. Eventually, we will test different HetR modified forms in *in vitro* transcription assays of heterocyst-specific genes.

We initiated our biochemical studies of HetR by obtaining rabbit anti-HetR antiserum using glutathione-column purified recombinant GST-HetR. This protein was produced from a plasmid obtained from Prof. J. Zhao, Peking University. Immunoblot analysis of whole-cell extracts from *Anabaena* showed a specific band of the expected size of ~35 kDa and no cross-reacting bands.

The shift to a more acidic pI after nitrogen step-down suggests that HetR might undergo phosphorylation. Interestingly, DIPHOS software detected a possible phosphorylation site at serine-179, which is essential for HetR function *in vivo* (3) and auto-proteolytic activity *in vitro* (10). To identify the HetR modifications, we planned to use 2D-PAGE followed by mass spectrometry of peptides (MALDI-MS) (performed by the Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University). However, we found that the majority of HetR from *Anabaena* French-press lysates is in the low-speed-centrifugation pellet fraction, which is unsuitable for IEF gels. This problem persisted after trying a variety of lysis buffers including the addition of nonionic detergents and DNAse treatment. However, we found that immunoblots of modified 1D SDS-PAGE (12% 37.5:1 acrylamide:bis-acrylamide) probed with anti-HetR antibody resolved a total of 4 bands of about 35-40 kDa, which presumably represent the different modified forms of HetR (Fig. 3). This separation approach will be continued in preparation for characterizing the HetR modifications by MALDI-MS.

Yeast two-hybrid experiment

Yeast two-hybrid constructs have been made to test for interactions among HetR, PatS, PatA, HetF, and Asr1734. We are using the Clontech Matchmaker Two-Hybrid System 3. Each *Anabaena* ORF is being cloned into both bait (pGBKT7) and prey (pGADT7) vectors. We have completed constructs for HetR, HetF, PatA, and Asr1734; the PatS constructions are in progress. We will also construct libraries of *Anabaena* genomic fragments as preys. We have just begun to test for some of the interactions. Our preliminary results suggest that Asr1734 may self-associate.

nifH-gfp reporter constructions

The culmination of heterocyst differentiation is the expression of *nif* genes and the resulting nitrogen-fixation activity. As stated in the introduction, little is known about the control of *nif* gene expression in cyanobacteria. The absence of *rpoN*, encoding sigma-54, or *nifA* shows that heterocysts must use a novel mechanism for regulating the *nif* genes compared to other well-studied bacteria. We plan to identify transcriptional regulators of *Anabaena nif* genes. The *nifHDK* operon is expressed exclusively in mature heterocysts and encodes nitrogenase. The *nifB-fdxN-nifS-nifU* operon is upstream and in the same orientation as the *nifHDK* operon. To study the transcriptional regulation of the *nifHDK* operon and to provide a genetic tool, we made transcriptional fusions of the upstream region of *nifH* to *gfp*. Our first construct contained 247 bp of the intergenic region between *nifU* and *nifH* fused to *gfp*. All of our shuttle-vector constructions are confirmed by DNA sequencing. However, no *nifH*-driven GFP expression was seen in heterocysts induced in liquid cultures and plates. A second construct, which contained 350-bp *nifH* upstream sequence, showed no expression of *nifH*-GFP when induced. A third construct contained 1 kb of the upstream region of *nifH* fused to *gfp*. When induced, this last construct showed strong expression in both vegetative cells and heterocysts. This result is consistent with a previous observation that showed vegetative-cell expression from a 700-bp *nifH* upstream sequence on a reporter plasmid (1). This raises the possibility that the *nifH* gene is kept off in vegetative cells by the action of a specific repressor or chromatin structure that is unable to repress expression of the promoter on a multi-copy-number plasmid. Differences in DNA topology on plasmids versus the chromosome may affect regulation.

Although consistent with some published results, our results are inconsistent with others. A 1-kb fragment containing the *nifH* promoter driving expression of a *luxAB* reporter on a plasmid showed heterocyst-specific expression (4). And, a 300-bp *nifH* promoter fragment driving expression of a *nifHDK* operon on a plasmid complemented the diazotrophic growth defect of the *nifH*-deletion mutant strain LW-1, but did not express a *cat* gene in vegetative cells (1). However, there are numerous differences among these experiments from various labs that could be responsible for the different results.

GFP fluorescence requires activation by oxygen, which could explain the negative results stated above because heterocysts are microoxic. However, several *gfp* reporter strains show GFP fluorescence in mature heterocysts including constructs from our lab containing the promoters: *patS*, *hetR*, *asr1734*, *hglE*, and the 1000-bp *nifH* fragment, among others. Also, we have immunoblot data showing an absence of GFP protein in induced filaments containing the 350-bp *nifH* upstream sequence driving *gfp*. Clearly, the issue has not been resolved, but we are hopeful that these data will lead us to a better understanding of the mechanisms that regulate expression of the *nif* genes. In the "proposed research" section I describe a much more straightforward biochemical approach to study expression of the *nifH* promoter, but we also plan specific *in vivo* experiments that build on the *gfp* reporter results.

Transcriptional regulation of hglE

The *hgl* genes code for products that form the heterocyst-specific glycolipid layer, which is required for maintaining a microoxic environment in heterocysts. The *hgl* genes are expressed at an

intermediate stage of differentiation, about the time when cells become committed to complete differentiation. To determine the mechanism controlling the transcription of the *hgl* genes, we constructed a plasmid containing the *hglE* promoter fused to *gfp*. Heterocyst-specific expression of *hglE-gfp* was observed in the reporter strain. The reporter strain will be used in experiments to characterize signaling and regulatory pathways that produce the developmental switch that commits a vegetative cell to complete differentiation into a heterocyst.

RNA polymerase purification and in vitro transcription assays

We have constructed His-tagged versions of the *Anabaena* RNAP beta (*alr1594*) and beta' (*alr1596*) subunits in shuttle vectors for experiments designed to isolate RNAP holoenzyme from differentiating cells. We will use the RNAP in *in vitro* transcription assays to identify the *cis*-acting sequences and transcription factors required for expression of heterocyst-specific genes. Our preliminary results are described in the "proposed research" section.

Asr1734

Extra copies of the novel gene *asr1734* inhibited heterocyst formation in several strains including the WT and two strains that form multiple contiguous heterocysts: a PatS null mutant and a *hetR*(R223W) mutant. Overexpression of *asr1734* also caused bleaching of autofluorescence in vegetative cells throughout filaments after nitrogen step-down. Unlike the WT, an *asr1734*-knockout mutant formed 5% heterocysts after a nitrogen-source shift from ammonium to nitrate (WT forms none) and 15% heterocysts with a weak Mch phenotype after step-down to medium lacking combined nitrogen. After nitrogen step-down, the *asr1734*$^{-}$ mutant had higher than normal amounts of *ntcA* messenger RNA. Apparent orthologs of *asr1734* were found only in two other filamentous nitrogen-fixing cyanobacteria, *Anabaena variabilis* and *Nostoc punctiforme*. A GFP reporter driven by the *asr1734* promoter, $P_{asr1734}$-*gfp*, was expressed specifically in differentiating heterocysts after nitrogen step-down (Fig. 4). Strains overexpressing *asr1734* and containing P_{hetR}-*gfp* or P_{patS}-*gfp* reporters failed to show normal patterned upregulation after nitrogen step-down, suggesting that *asr1734* is involved in the earliest steps of the heterocyst differentiation pathway.

Sigma factors

In *Anabaena*, genes encoding five group 2 sigma factors have been characterized: *sigB*, *sigC*, *sigD*, *sigE*, and *sigF*. None of these have been shown to be heterocyst specific, required for growth, or necessary for development. It is likely that there is functional redundancy among the group 2 sigma factors as well as with the principal group 1 sigma factor (6, 8), such that individual promoters can be recognized by more than one sigma factor.

To determine if the sigma factor genes are expressed in differentiating cells, we used transcriptional fusions with *gfp* for eight sigma-factor genes. Half of these are unpublished sigma factor genes (*sigI*, *sigJ*, *sigK*, and *sigL*) and the others have been previously published (*sigC*, *sigD*, *sigE*, and *sigF*) (2, 8). Fluorescence microscopy studies revealed that, after nitrogen step-down, the expression of *sigC*, *sigF*, and *sigL* increased only in proheterocysts and heterocysts. Time-lapse microscopy studies are currently underway to determine the spatial and temporal pattern of expression for *sigC*, *sigF*, and *sigL*. These time-course data will help determine whether the potential targets for these three sigma factors are early, middle, or late genes.

Oligopeptide permeases

These experiments are related to our efforts to understand how HetR activity is controlled. We are interested in how PatS, made in differentiating cells, inhibits HetR in neighboring cells. One hypothesis is that PatS is exported from differentiating proheterocysts, presumably into the periplasmic space, and then imported into adjacent vegetative cells where it inhibits their differentiation by interacting with HetR. The import of peptides depends on oligopeptide permeases

that consist of multisubunit ABC-transporters and periplasmic oligopeptide-binding proteins. Three genes coding for potential oligopeptide-binding proteins are present in the *Anabaena* genome, and we are attempting to inactivate them in all possible combinations to determine whether the mutant strains show defects in heterocyst pattern formation. Disruption by single recombination of *alr3762* ("*opp1*") and *alr0140* ("*opp2*") yielded strains that did not differ from the WT, while inactivation of *alr3884* ("*opp3*") has been problematic for unknown reasons. Among three independent presumptive *opp3* single recombinants examined, one clone showed unimpaired diazotrophic growth and normal heterocyst pattern, but was resistant to inhibition by PatS-5, while two others failed to grow in liquid combined-nitrogen-free medium when antibiotic selection was maintained. They initially formed apparently normal heterocysts, but then the cultures bleached, and the heterocysts accumulated huge cyanophycin granules. It is premature to speculate on the nature of these different but interesting phenotypes until the genotypes of presumptive mutants are confirmed.

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