August 5, 2004

Dear James E. Tavares,

This is our final progress report for Grant No. DE-FG03-98ER20309.

The following paper resulting from our studies was recently submitted to PNAS.

Khudyakov, I. Y. and J. W. Golden. Different functions of HetR, a master regulator of heterocyst differentiation in *Anabaena* sp. strain PCC 7120, can be separated by mutation. Submitted August 2, 2004

An additional paper based on the research supported by this grant is currently being prepared. The authors and preliminary title are as follows.

Khudyakov, I. Y., D. W. Lee, R. Mella, and J. W. Golden. Group 2 sigma factors of *Anabaena* sp. strain PCC 7120 acquired through horizontal gene transfer.

The following three sections present our recent work on sigma factors and *hetR*. Our recent findings have caused us to shift our research plan to emphasize studies of *hetR* because they are more clearly important for the control of heterocyst development. Our studies of sigma factors and related genes have shown that they are not a central control mechanism for heterocyst development, and shows that the paradigm of a sigma factor cascade established for spore formation in *Bacillus* sp. is not applicable to heterocyst development. Our current work on *hetR* is providing important insights into the control of pattern formation, and will provide the basis for future work on the signaling pathways that control cyanobacterial heterocyst development.

Plasmid-borne group 2 sigma factors.

Most fresh water and terrestrial strains of cyanobacteria studied so far share a common complement of four group 2 sigma factors. *Anabaena* sp. strain PCC 7120, *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* strain ATCC 29133 possess additional group 2 sigma factors that form a separate loose phylogenetic cluster and are much more diverse than the highly similar *Anabaena* and *Nostoc* representatives from the four common subgroups. While gene context is conserved for *Anabaena* and *Nostoc* group 2 sigma factor genes from the common subgroups, no such conservation can be found for the additional sigma factor genes. These data suggested that sigma factor genes from this subgroup have been acquired through lateral transfer.

In *Anabaena* sp. strain PCC 7120 the additional group 2 sigma factors reside on two megaplasmids: *sigG* on the alpha plasmid and *sigB* and *sigH* on the beta plasmid. These sigma factors, similar to the four chromosomal group 2 sigma factors, were inactivated and proved dispensable for heterocyst production and diazotrophic growth. To test the possibility of curing *Anabaena* PCC 7120 of the megaplasmids carrying the sigma factor genes, the *sacB* positive selection cassette on pRL277 was employed. Double mutant strains obtained with pAM2179 (Em^r) and pRL277 (*sacB*-Sp^r Sm^r) suicide vectors integrated into *sigB* and *sigH* genes, respectively, were plated on sucrose-containing plates without antibiotic selection. Among several dozen sucrose resistant clones that were tested for antibiotic resistance, two clones showed concurrent loss of the second resistance marker. PCR and Southern blot analysis showed that in these two clones the beta plasmid has been lost. An attempt to cure *Anabaena* PCC 7120 of the alpha megaplasmid using the same strategy have failed, pointing to the possibility that this plasmid could be essential for growth and thus represent a minichromosome. Interestingly, *sigG*

on the alpha plasmid has a close ortholog in *Anabaena variabilis* ATCC 29413, which also contains large regions homologous to regions of the PCC 7120 alpha plasmid, sharing the same genes and conserved gene order. Related sequences are not found in the genome of *Nostoc punctiforme* strain ATCC 29133.

As we reported previously, strains cured of the beta plasmid grew well in both nitrogen-replete and nitrogen-free media, but were sensitive to lower concentrations of Cu^{+2} and Zn^{+2} than the wild type, confirming our suggestion that several clusters of genes on this plasmid are involved in heavy metal resistance. Heavy metal resistance determinants are among the most common traits found on a variety of bacterial plasmids. An additional feature of the two cured strains is their poor expression of at least several genes and *gfp*-reporters introduced on pDU1- and RSF1010-based shuttle vectors. Currently we do not know if this is a copy number-related effect, or inefficient expression of plasmid-borne genes due to the loss of sigma factors and other transcription factors residing on the beta plasmid.

The presence of group 2 sigma factors is an unusual feature of cyanobacterial megaplasmids. The adaptive value of newly acquired sequences must depend on appropriate and coordinated regulation with the rest of the host genome. It appears that the acquisition of cyanobacterial sigma factors is one way that large plasmids circulating in cyanobacterial populations control their gene expression.

HetR

To better understand the interactions among regulatory proteins during heterocyst differentiation and pattern formation, we attempted to isolate and identify extragenic suppressors of the negative regulators PatS and HetN. Several strains that were able to bypass inhibition of heterocyst differentiation caused by these negative regulators and that showed distorted heterocyst pattern were isolated, and the genetic lesions were localized to the *hetR* gene. A detailed characterization of one of these mutants, which contains a *hetRR223W* allele, is in a submitted paper with the following abstract.

Abstract

The HetR protein has long been recognized as a key player in the regulation of heterocyst development, and its role in heterocyst pattern formation has been proposed, but never proven. HetR is known to possess autoproteolytic and DNA-binding activities. During a search for mutants of *Anabaena* sp. PCC 7120 that can overcome heterocyst suppression caused by overexpression of the *patS* gene, which encodes a negative regulator of differentiation, a bypass mutant strain, S2-45, was isolated that produced long and irregularly spaced strings of multiple contiguous heterocysts (Mch phenotype) in combined nitrogen-free medium. Analysis of the S2-45 mutant revealed a R223W mutation in HetR, and reconstruction in the wild-type background showed that this mutation was responsible for the Mch phenotype and resistance not only to overexpressed *patS*, but also overexpressed *hetN*, another negative regulator of differentiation. Ectopic overexpression of the *hetRR223W* allele in the *hetRR223W* background resulted in a conditional lethal (complete differentiation) phenotype. Analysis of heterocyst pattern in the *hetRR223W* mutant revealed that heterocysts differentiate randomly along filaments, indicating that a single mutation in *hetR* can apparently block all signals governing heterocyst pattern formation. These data provide direct genetic evidence that apart from being an essential activator of the differentiation process, HetR is a central regulator of heterocyst pattern formation, and that these functions can be separated by mutation.

Oligopeptide permeases

Current models of heterocyst pattern formation assume the existence of a diffusible inhibitor of differentiation that interacts with a non-diffusible positive regulator, and PatS peptide or a product of its cleavage was proposed to function as such an inhibitor. In this model, PatS must diffuse or be actively exported from (pro)heterocysts, presumably into the periplasmic space, and then imported into adjacent vegetative cells where it inhibits their differentiation. 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* PCC 7120 genome, and we attempted to inactivate them and check whether mutant strains will be resistant to exogenous PatS-5 synthetic peptide, overexpression of *patS*, and show any defect in heterocyst pattern formation. Disruption by single recombination of *alr3762* ("*opp1*") and *alr0140* ("*opp2*") yielded strains that did not differ from the wild type, while presumptive inactivation of *alr3884* ("*opp3*") (not confirmed by PCR or Southern blotting yet) yielded strains with seemingly inconsistent phenotypes. Among three independent 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, but one possible explanation might involve partial redundancy in the specificity of different oligopeptide transport systems and different levels of their expression in different mutant clones.