Systems level approaches to understanding and manipulating heterocyst differentiation in *Nostoc punctiforme*: sites of hydrogenase and nitrogenase synthesis and activity.

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Project Goals: Heterocyst-forming cyanobacteria, such as *Nostoc punctiforme*, are applicable for cost-effective photo-biohydrogen production, providing the frequency of heterocysts in the filaments can be increased by genetic manipulation and the metabolic end product, H_2, is uncoupled from growth. The goals of this project are to apply transcriptomic and proteomic analyses to free-living and symbiotically grown wild type and mutants strains to identify regulatory elements and pathways that can be manipulated.

We hypothesize that if the frequency of heterocysts, sites of nitrogen fixation and hydrogen evolution, can be increased approximately 3-fold above the less than 10% normally found in filaments, then heterocyst-forming cyanobacteria would be applicable for cost effective photo-biohydrogen production. In *Nostoc punctiforme*, the heterocyst frequency of 8% in the free-living state is increased to 30-35% when in symbiotic association with terrestrial plants, such as the hornwort *Anthoceros* spp. and the angiosperm *Gunnera* spp. There is an increase in symbiotic nitrogen fixation that parallels the increased heterocyst frequency and about 85-90% of the metabolic end product, ammonium, is excreted to the plant partner. This is the kind of metabolic uncoupling we seek for biohydrogen production. We model heterocyst differentiation in the context of establishing the pattern of heterocyst spacing, maintaining the pattern during growth on N_2 and symbiotic disruption of the spacing pattern. Establishment of the pattern following combined nitrogen deprivation is further modeled as a two stage process: biased initiation where a cluster of cells sense nitrogen starvation and initiate the differentiation process, followed by competitive resolution through interactions between HetR and PatS, where a single cell in the cluster completes the differentiation and maturation into a mature functional heterocyst. We do not know whether the same or similar regulatory networks are involved in establishment, maintenance and disruption of the pattern. We are applying transctriptomic and proteomic analyses with wild-type and mutant strains to identify the regulatory circuits of free-living heterocyst differentiation and how those circuits may have been co-opted during symbiotic growth.

Genetics. We have isolated an exogenously induced transposon mutant of *N. punctiforme* that displays a heterocyst frequency (> 30% of the total cells) and spacing pattern (multiple singular heterocysts) that is essentially the same as that in symbiotic association. This is the heterocyst frequency and spacing pattern we hypothesize would be an experimental platform for enhanced hydrogen production. The mutant does grow with N_2 as the sole nitrogen source, but at a slower rate than the wild type and with about 30% of the rate of nitrogen fixation (acetylene reduction). Thus, the mutant physiology does not parallel that of the symbiotic growth state. The targeted gene encodes a protein unique to heterocyst-forming cyanobacteria that we have designated PatN. PatN has four distinct domains: a 30 amino acid cytoplasmic region, a transmembrane domain, a glutamine rich coiled-coil domain in the periplasm, and a signal sequence. Our working hypothesis is that PatN may be involved in the translocation of a negative acting

heterocyst differentiation element, such as, for example, the RGSGR C-terminal pentapeptide of PatS. We are setting up a microscope for fluorescence recovery after photobleaching (FRAP) analysis to examine translocation properties of the mutant relative to the wild type. The RGSGR pentapeptide is also present in HetN, which is modeled to be involved in maintenance of the heterocyst spacing pattern. The putative HetN protein in *N. punctiforme* lacks the RGSGR motif. Bioinformatic analysis revealed two additional proteins encoded by *N. punctiforme* as containing the RGSGR motif. Single deletion mutants do not result in a multiple contiguous heterocyst (Mch) spacing pattern as is characteristic of *hetN* mutants of *Anabaena* sp. strain PCC 7120. Moreover, *patS* deletion mutants of *N. punctiforme* do not show an extensive Mch pattern. These results imply considerably more cross-talk or complementation in negative acting regulatory elements in *N. punctiforme* compared to *Anabaena* 7120. To confirm this suggestion we are constructing multiple deletion mutants.

Transcriptomics. To more rigorously analyze time course microarray data, we have adapted the Bayesian Analysis of Time Series (BATS) software. BATS analyses have reduced the number of statistically significant genes that are differentially expressed during heterocyst differentiation in the wild type and mutant strains. The reanalysis confirmed that the wild type (511 genes) and mutants impaired in HetR (1150 genes) and HetF (722 genes) (two positive acting regulatory elements) function have different patterns and numbers of genes differentially transcribed during nitrogen starvation. We interpret these data to verify that the primary role of HetR and HetF, directly or indirectly, is to influence gene transcription. Conversely, other than *patN* itself, no other genes are altered in their patterns of differential transcription in the *patN* mutant. This observation implies that PatN has a structural or functional as opposed to transcriptional role in the differentiation of heterocysts and supports the FRAP analysis above to define that role.

Proteomics. We have completed three replicates of three dimensional SDS-PAGE and LC fractionation of N_2-grown, wild type *N. punctiforme* and protein identification by MudPit MS/MS. These analyses yielded 1,210 identified proteins in the membrane (40,000 x g pellet), molecular complex (150,000 x g pellet) and soluble (150,000 x g supernatant) fractions. Known heterocyst structural and catalytic proteins were present in all fractions. We have completed two replicates of symbiotic colonies isolated from the hornwort *Anthoceros punctatus.* Due to the small amount of biomass, the symbiotic cell extracts were not subjected to centrifugal fractionation. These analyses yielded only 306 identified proteins. However, the identified proteins were highly enriched in cell envelope, energy metabolism and transporters. Present were PsbB and PsbC, but not PsbA, which may account for the lack of photosynthetic oxygen evolution in heterocysts and the lower rate of symbiotic CO_2 fixation by *N. punctiforme*. We are currently exploring methods to fractionate the small-volume, symbiotic cell extracts for better resolution of less abundant proteins.