Chapter 16
Better Living Through *Cyanothece* – Unicellular Diazotrophic Cyanobacteria with Highly Versatile Metabolic Systems

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Abstract *Cyanothece* sp. ATCC 51142 is a unicellular, diazotrophic cyanobacterium with a versatile metabolism and very pronounced diurnal rhythms. Since nitrogen fixation is exquisitely sensitive to oxygen, *Cyanothece*utilizes temporal regulation to accommodate these incompatible processes in a single cell. When grown under 12 h light–dark (LD) periods, it performs photosynthesis during the day and N_2 fixation and respiration at night. Genome sequences of *Cyanothece* sp. ATCC 51142 and that of five other *Cyanothece* species have been completed and have produced some surprises. Analysis at both the transcriptomic and the proteomic levels in *Cyanothece* sp. ATCC 51142 has demonstrated the relationship of the metabolic synchrony with gene expression and has given us insights into diurnal and circadian regulation throughout a daily cycle. We are particularly interested in the regulation of metabolic processes, such as H_2 evolution, and the way in which these organisms respond to environmental cues, such as light, the lack of combined nitrogen, and changing O_2 levels. *Cyanothece* strains produce copious amounts of H_2 under different types of physiological conditions. Nitrogenase produces far more H_2 than the hydrogenase, in part because the nitrogenase levels are extremely high under N_2-fixing conditions. With *Cyanothece* 51142 cultures grown in NO_3-free media, either photoautotrophically or mixotrophically with glycerol, we have obtained H_2 production rates over 150 μmol/mg Chl/h.

16.1 Introduction

Unicellular, diazotrophic cyanobacteria are interesting and versatile organisms. They perform oxygenic photosynthesis, but they can also fix atmospheric N_2. Nitrogenase, the enzyme that is responsible for this N_2 fixation into ammonia is

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normally rapidly and irreversibly inactivated upon exposure to molecular oxygen. Thus, one interesting question about such organisms is how they regulate nitrogenase activity in the presence of poisonous oxygen. In addition, what other properties do they have that might make them valuable for other kinds of experimentation? We have been interested in studying these organisms and in understanding how they regulate various metabolic properties. Most recently, we have also been interested in the ability of these organisms to use light energy to generate alternative biofuels such as H_2.

We have primarily studied the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142, hereafter *Cyanothece* 51142 (Reddy et al. 1993; Schneegurt et al. 1994), which performs photosynthesis during the light and fixes nitrogen during the dark (Schneegurt et al. 1997a, b, 1998). Our early studies with *Cyanothece* 51142 showed a strong correlation between activity and transcript level for a subset of genes related to photosynthesis (Meunier et al. 1998) and N_2 fixation (Colón-López et al. 1997) during 12 h light–dark (LD) cycles. In addition, differential gene expression was reported for the main photosynthetic genes and the nitrogenase genes in the light and dark, respectively (Colón-López et al. 1997, 1999; Colón-López and Sherman 1998). During a diurnal period, *Cyanothece* 51142 cells actively accumulate and degrade different storage inclusion bodies for the products of photosynthesis and N_2 fixation. This ability to utilize metabolic compartmentalization and energy storage makes *Cyanothece* an ideal system for bioenergy research and for studies on how a unicellular organism balances multiple, often incompatible, processes in the same cell. The genome sequences will help provide a significant basis for future insights into this metabolic "balancing act".

Recently, whole genome microarray experiments were carried out to determine the diurnal gene expression under LD conditions (12 h L/12 h D) (Stöckel et al. 2008) and under continuous light (LL) (Toepel et al. 2008). Both groups found ~30% of the ~5000 genes on the microarray exhibited diurnal oscillations under 12 h LD conditions and Toepel et al. (2008) demonstrated that ~10% of the genes demonstrated circadian behavior during growth in free-running (LL) conditions. Toepel et al. (2008) also demonstrated that nitrogenase transcript abundance and nitrogenase activity were correlated in *Cyanothece* 51142 under LL and that N_2 fixation followed a ~24 h rhythm under these conditions, albeit with reduced rates. Such results indicate a LD-independent expression pattern for nitrogenase genes, consistent with the circadian behavior for nitrogenase-related genes suggested by Sherman et al. (1998).

Photosynthetic activity depends on incident light, although genes encoding photosynthetic proteins can display a diurnal or a circadian-dependent expression pattern (Stöckel et al. 2008; Michael et al. 2008; Toepel et al. 2009). In *Cyanothece* 51142, maximum photosynthetic rates in a LD cycle occurred after 6–8 h light incubation and photosynthetic capacity decreased strongly during the N_2 fixation period (Meunier et al. 1998; Toepel et al. 2008). Toepel et al. (2008) showed that photosynthetic rates were lower during LL growth and demonstrated no circadian-related pattern for photosynthesis genes. In the case of growth under continuous light, the glycogen content stayed at high levels and did not decrease until the cells were

again placed in darkness (Toepel et al. 2008). Furthermore, these results indicated that nitrogenase transcription and activity was metabolically or energetically regulated via glycogen breakdown and suggested that photosynthesis is light activated, but probably regulated by the internal carbohydrate level.

The work with *Cyanothece* 51142 has provided some answers as to the regulation of the disparate metabolic processes, but also has led to some surprises. These include the discovery of a small linear chromosome that coexists along with the larger, circular chromosome. In order to better understand the genomic basis of the genus *Cyanothece*, an additional six *Cyanothece* strains have been sequenced by the Department of Energy Joint Genome Initiative. We have begun to compare and contrast the different genomes and have found a *Cyanothece* gene core within this genus using five of the strains so far sequenced. Interestingly, *Cyanothece* 7425 demonstrates significant differences in many genomic properties. We will discuss some of the important attributes of gene regulation that we have determined in *Cyanothece* 51142 as well as discuss key features in regard to H_2 production in these strains.

16.2 Results

16.2.1 Genome Sequencing of Cyanothece 51142

The genome of *Cyanothece* 51142 was sequenced at the Washington University Genome Sequencing Center (St. Louis, MO), and the finished assembly was independently confirmed using an optical restriction map generated by OpGen, Inc. (Welsh et al. 2008). The 5.5 Mb *Cyanothece* 51142 genome consists of a 4.93 Mb circular chromosome, four plasmids ranging in size from 10 to 40 kb, and notably a 430 kb linear chromosome. The finding of a linear element in the *Cyanothece* genome was unanticipated, but was confirmed by two independent genome assembly approaches (Welsh et al. 2008). This also represented the first report of a linear element in the genome of a photosynthetic bacterium, although linear genomic elements have been identified in other bacterial genera, such as *Borrelia* (Ferdows and Barbour 1989), *Streptomyces* (Kinashi et al. 1992), and *Agrobacterium* (Allardet-Servent et al. 1993).

The gene content of the linear chromosome was examined to investigate its possible origin and importance to the organism. Several genes are found on both the circular and the linear chromosomes, including a *coxABC* operon and a cluster containing genes related to glycolysis and fermentation (*ppk*, *pyk*, *pgi*, *eno*, *ackA*, *glgP*). This cluster on the linear chromosome contains the only gene that encodes an L-lactate dehydrogenase, required for the terminal step in lactate fermentation, and suggests that the linear chromosome may play a role in fermentation. Additional genes unique to the linear chromosome are ones encoding the integrase–recombinase protein XerC, an *xseA/xseB* operon, and a *hicA/hicB* operon. Most of the remaining genes on the linear chromosome are either hypothetical,

unknown, or of uncertain function. The mechanisms of maintenance and replication of the linear chromosome remain unknown, and sequence analysis did not indicate the presence of any feature, such as inverted repeats or stem–loop structures, known to be related to these functions in previously characterized genera (Volff and Altenbuchner 2000).

The predicted proteome of *Cyanothece* consists of 5269 open reading frames: 34% of known function, 29% of uncertain function, and 37% of unknown/hypothetical (Welsh et al. 2008). The annotation of genes of unknown function was greatly aided by data from the high-throughput proteomic analysis. Proteomic data were used, in conjunction with early draft genomic sequence data, to build an accurate mass and time (AMT) library (Lipton et al. 2002) for use in quantitative proteomic experiments. The combined analysis of proteome and genome data is an important approach that resulted in the inclusion or reclassification of 510 genes and lent an additional level of validation to the genome annotation. The importance of the proteomic data to metabolic objectives will be discussed in later sections.

16.2.2 Nitrogenase Genes

In *Cyanothece*, most of the genes involved in nitrogen fixation are located in a single contiguous cluster containing 28 genes separated by no more than 3 kb, with conserved synteny to those found in most other sequenced nitrogen-fixing cyanobacteria (Fig. 16.1). The cluster is more distantly conserved in *Trichodesmium erythraeum* sp. IMS 101, where 20 genes are present in a single large cluster. In the heterocyst-forming *Anabaena* and *Nostoc* strains, one or more inserts ranging in size from 9 to 24 kb break the cluster into several smaller clusters, with several genes duplicated or missing between clusters. The conserved synteny of the genes within the *nif* clusters of the *Cyanothece* and *Anabaena* families, together with the proteome-wide phylogenetic tree, supports a single acquisition event of the *nif* cluster in a common ancestor. The *Synechococcus* sp. JA-2-3B′a(2-13) and *Synechococcus* sp. JA-3-3Ab strains contain a single contiguous cluster of 20 genes, but the order and orientation of the genes are extensively reorganized relative to that seen in the other strains. *Cyanothece* 51142 contains the largest contiguous cluster of nitrogen fixation-related genes yet observed in cyanobacteria. If nitrogen fixation

Fig. 16.1 Clusters of N_2 fixation-related genes. Shown are genes with conserved synteny between *Cyanothece* 51142 and other nitrogen-fixing cyanobacteria. *Black arrows* represent genes assigned to functional categories and *white arrows* correspond to hypothetical genes and genes of unknown function. A possible inversion event in *Synechococcus*sp. JA-3–3Ab is highlighted in brackets. GenBank accession numbers for the sequences used are as follows: *Cyanothece*sp. ATCC 51142, CP000806; spheroid body of *Rhopalodia gibba*, AY728387; *Crocosphaera watsonii*WH 8501,

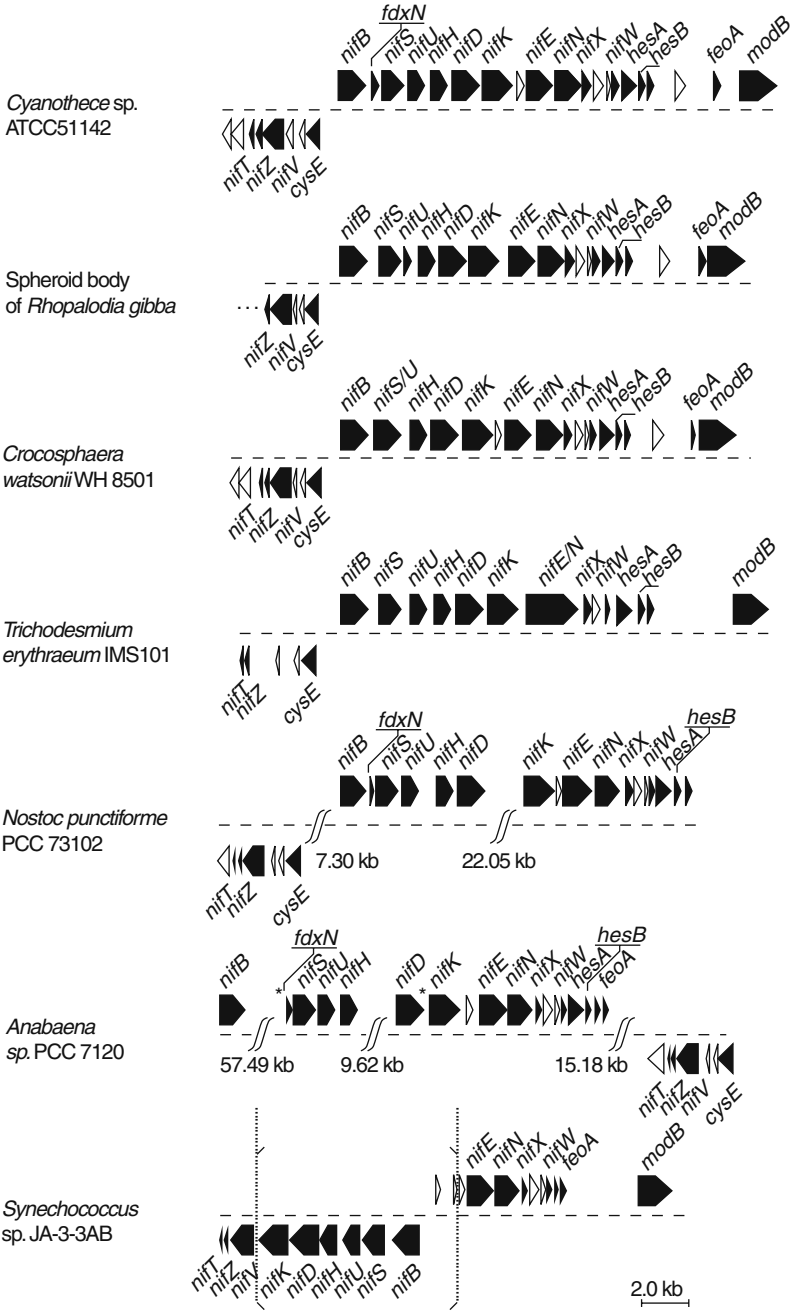


Fig. 16.1 (continued) AADV02000024; *Trichodesmium erythraeum*IMS101, CP000393; *Nostoc punctiforme*PCC 73102, CP001037; *Anabaena*sp. PCC 7120, BA000019; and *Synechococcus*sp. JA-3–3Ab, CP000239. Reproduced from PNAS 2008, vol 105:15094 with permission of the publisher

was acquired in a single ancient event, this single contiguous cluster may resemble that seen in the original ancestor. The smaller cluster present in the thermophilic *Synechococcus* strains either underwent significant gene loss and rearrangement from a single common ancestor common to all diazotrophic cyanobacteria or was acquired in a separate event by lateral gene transfer from a non-cyanobacterial organism. In either case, based on the phylogenetic evidence, nitrogen fixation was apparently present early in the evolutionary history of cyanobacteria and was subsequently lost in the non-diazotrophic β-cyanobacterial strains.

16.2.3 Metabolic Compartmentalization

Intracellular compartmentalization provides a strategy for *Cyanothece* cells to tightly regulate storage of metabolic products. The *Cyanothece* genome data confirmed the details of the pathways of storage granule accumulation and degradation by elucidation of the genes involved, provided insights into interconnections between different pathways, and highlighted the central role of nitrogen fixation in these organisms (Welsh et al. 2008). Certain filamentous nitrogen-fixing cyanobacterial strains separate photosynthesis and nitrogen fixation spatially by the differentiation of a subset of cells into heterocysts, which fix nitrogen and do not perform oxygenic photosynthesis (Haselkorn 1978; Wolk 1996). However, non-heterocyst-forming unicellular nitrogen-fixing cyanobacteria, such as *Cyanothece*, must separate these processes temporally by performing photosynthesis during the day and fixing nitrogen during the night (Sherman et al. 1998). Nitrogen fixation is an energy-intensive process requiring the use of 16 ATP molecules per molecule of N_2 converted to ammonia (Dean, Bolin and Zheng 1993). A respiratory burst at the beginning of the dark period provides some of the energy required for nitrogen fixation and serves to further deplete cellular O_2 which would otherwise inhibit nitrogen fixation. Photosynthetic capacity is also at a minimum during this time (Sherman et al. 1998), which further protects nitrogenase from oxygen damage. Fixed nitrogen is stored in cyanophycin granules, which are fully depleted during the next light period. Carbohydrates produced from photosynthesis are stored during the day in glycogen granules, which are rapidly consumed early in the dark period as a substrate for respiration (Sherman et al. 1998). Interestingly, two distinctly different glycogen debranching enzymes are present in the *Cyanothece* genome, one that is found only in the β-cyanobacterial clade (*glgP*) and another (*glgX*) that is found mainly in the α-cyanobacteria. The presence of these two enzymes suggests that granule accumulation and degradation is important and carefully regulated. The circadian cycle in *Cyanothece* 51142 cells, once entrained, persists in continuous light (Colón-López and Sherman 1998) or continuous darkness (Schneegurt et al. 2000). The temporal regulation of metabolic processes is crucial to *Cyanothece* 51142, as each is involved in a different, but interrelated, aspect of cellular metabolism.

The *Cyanothece* genome contains all of the genes required for fermentation, including the production of ethanol and hydrogen, both of which require a low or

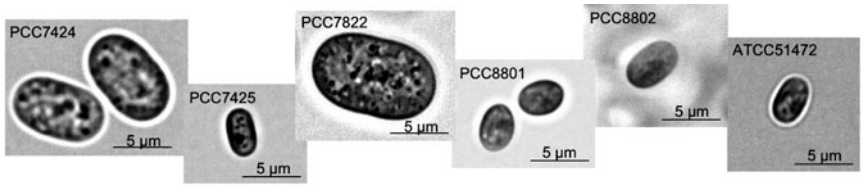
anoxic environment. *Cyanothece* 51142 creates such an intracellular environment during the early dark period in order to fix nitrogen. Stal and Moezelaar (1997) have proposed that 6-phosphogluconate dehydrogenase, present in the pentose phosphate pathway of *Cyanothece*, is primarily involved in fermentation in cyanobacteria and that its presence indicates fermentative capability. Therefore, *Cyanothece* has the ability to ferment glucose to ethanol and to produce hydrogen, and may carry out these processes early in the dark period in order to generate additional ATP molecules for nitrogen fixation. However, this same intracellular environment is generated in LL conditions (Toepel et al. 2008) and this may provide additional opportunities for N_2fixation and H_2 evolution.

16.2.4 Genomic Sequencing of Six Additional Cyanothece *Strains*

An additional six *Cyanothece* strains were sequenced by the Department of Energy Joint Genome Initiative as a first step toward understanding the nature of the *Cyanothece* genus (Table 16.1). The strains were isolated in different environments around the world and the size and shape of the organisms, as well as other important properties, can be seen in Table 16.1. All strains are capable of nitrogen fixation as well as mixotrophic growth on different carbon sources. The genome size of the strains varies from 4.6 to 6.4 Mb and none of these strains appear to have a linear chromosome.

The phylogenetic analysis of a broad grouping of cyanobacteria, including all of the sequenced *Cyanothece* strains, is shown in Fig. 16.2. It is evident that all

Table 16.1 Genomic information of six species of the genus *Cyanothece* sequenced by the Department of Energy Joint Genome Initiative. Reproduced from Welsh et al. (2008) by permission



STRAIN	PCC7424	PCC7425	PCC7822	PCC8801	PCC8802	ATCC51472
ORIGIN	Senegal	Senegal	India	Taiwan	Taiwan	Texas, USA
SIZE	7-8 µm	3-4 µm	10 µm	3-4 µm	4-5 µm	4-5 µm
MIXOTROPHIC GROWTH	yes	yes	yes	yes	yes	yes
PHYCOERYTHRIN	yes	no	yes	yes	no	no
N_2 – FIXATION	yes	yes	yes	yes	yes	yes
% GC	39%	51%	40%	40%	40%	40%*
GENOME SIZE	6.4 Mb	5.7 Mb	5.7 Mb	4.6 Mb	4.7 Mb	5.5 Mb*
GENE NUMBER	6107	5574	5266	4436	4681	*
COG GENES	3432	3345	3006	2705	2866	*

*Estimated or not yet determined

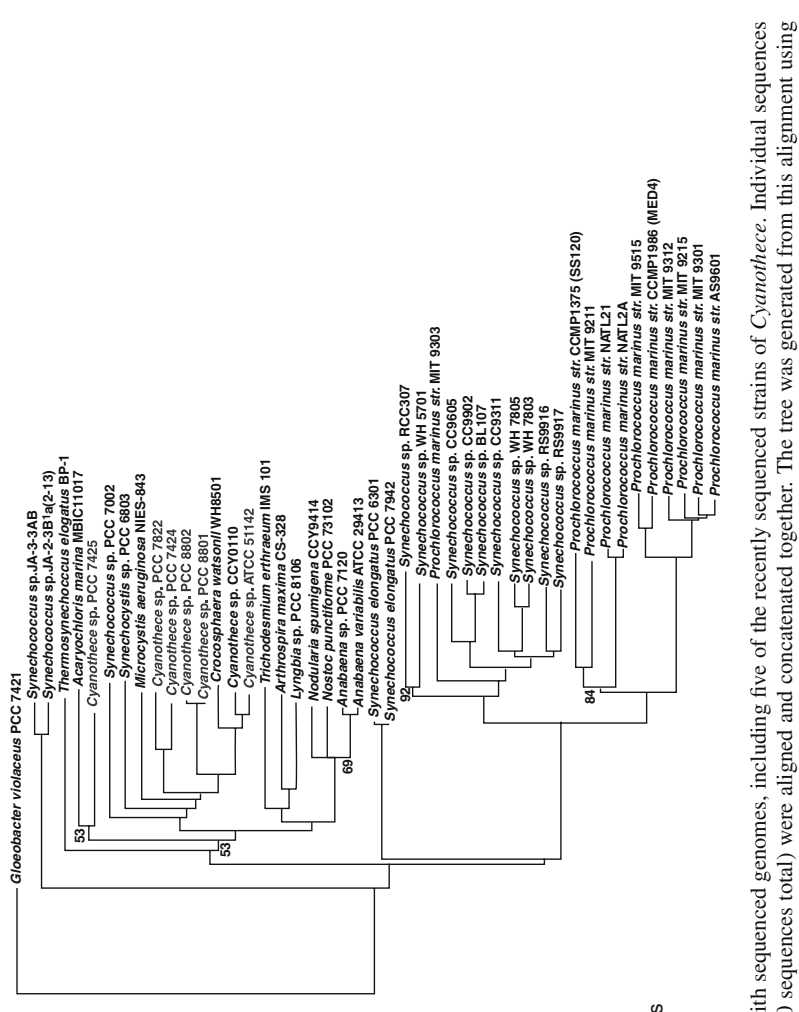


Fig. 16.2 Phylogenetic tree of cyanobacteria with sequenced genomes, including five of the recently sequenced strains of *Cyanothece*. Individual sequences from 375 groups of orthologous proteins (17250 sequences total) were aligned and concatenated together. The tree was generated from this alignment using the Fitch–Margoliash method. Bootstrap values for branches are 100 unless labeled otherwise. See Welsh et al. (2008) for details

of the *Cyanothece* strains, except for *Cyanothece* sp. PCC 7425, cluster closely together along with *Crocosphaera watsonii* WH8501. *Cyanothece* sp. PCC 7425 branches somewhat differently and is closer to *Acaryochloris marina* MBIC11017. Interestingly, this broad cluster includes a few non-nitrogen-fixing strains, such as *Synechocystis* sp. PCC 6803, which has many genes that show sequence similarity to those in *Cyanothece* sp. ATCC 51142. Such data again raise the question of whether or not *Synechocystis* once had nitrogen fixation genes that were subsequently lost or if *Cyanothece* acquired the genes at some later date. All of the *Cyanothece* strains so far sequenced have large clusters of nitrogenase genes, although not as complete as those in *Cyanothece* 51142. Once again, *Cyanothece* 7425 is the most divergent with the nitrogenase genes split into two medium-sized clusters. In addition, all the strains have clusters of the bidirectional hydrogenase genes (*hox*) (Fig. 16.3). *Cyanothece* 51142 has the most compact operon, whereas *Cyanothece* 7425 has the largest number of hypothetical genes within a larger cluster.

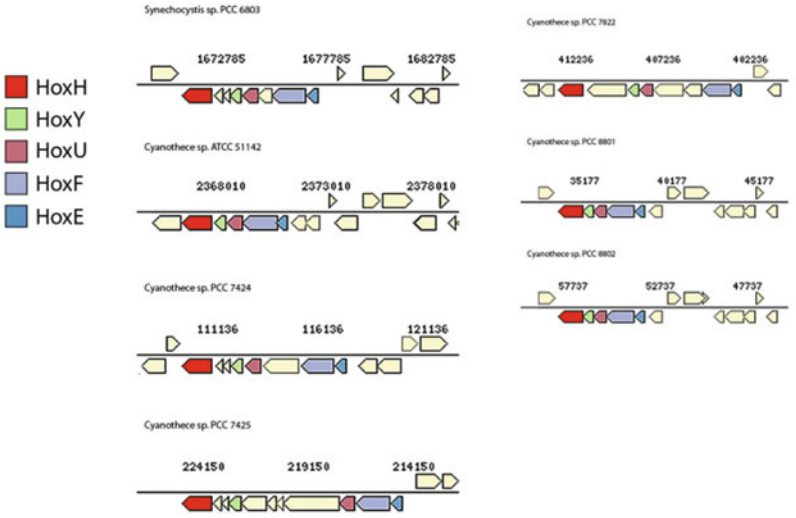


Fig. 16.3 Chromosomal organization of *hox* operons in seven strains of cyanobacteria, including *Synechocystis* sp. PCC 6803 and six *Cyanothece* strains. In all cases, the *hox* cluster encodes all of the proteins needed to produce a functional bidirectional hydrogenase

16.2.5 Genomics and Hydrogen Production

One objective for sequencing these genomes was to determine their capabilities for biofuels production. We were specifically interested in analyzing hydrogen production and to determine if all strains contain the genes for the three types of hydrogenases found in diazotrophic cyanobacteria: nitrogenase, uptake hydrogenase, and bidirectional hydrogenase. It has been demonstrated in *Anabaena* that mutants that lack the uptake hydrogenase evolve more hydrogen than the wild type

Table 16.2 Presence or absence of the three genes capable of hydrogen production in select cyanobacterial strains

Cyanobacterial strain	Nitrogenase	Bidirectional hydrogenase	Uptake hydrogenase
Cyanothece sp. ATCC 51142	+	+	+
Cyanothece sp. PCC 7425	+	+	–
Cyanothece sp. PCC 7822	+	+	+
Cyanothece sp. PCC 7424	+	+	+
Cyanothece sp. PCC 8801	+	+	+
Cyanothece sp. PCC 8802	+	+	+
Anabaena variabilis ATCC 29413	+	+	+
Anabaena sp. PCC 7120	+	+	+
Nostoc punctiforme PCC 73102	+	–	+
Synechocystis sp. PCC 6803	–	+	–
Trichodesmium erythraeum IMS101	+	–	+

(Dutta et al. 2005). Thus, we carefully analyzed the genomic sequences to determine if any of the strains were lacking an uptake hydrogenase system. In fact, all strains except *Cyanothece* 7425 had the uptake hydrogenase (Table 16.2). From this data, we were hopeful that *Cyanothece* 7425 might be a particularly important strain for hydrogen production. Of course, *Synechocystis* 6803 lacks an uptake hydrogenase, but it also lacks nitrogenase and produces relatively low levels of H_2 from the bidirectional hydrogenase. Thus, the presence or absence of the uptake hydrogenase is only one factor in determining the ultimate levels of hydrogen that can be produced.

16.2.6 Transcription and Translation of Hydrogen Production Genes

Another objective has been to understand transcription and transcriptional regulation of genes in *Cyanothece* 51142 under a variety of different environmental conditions. We are particularly interested in transcriptional regulation of N_2-fixing cells when grown under a variety of different light regimes. The most complete work to date has been on *Cyanothece* 51142, first using Northern blots for individual genes, and then using microarrays for full genome transcriptional analyses (Colón-López and Sherman 1998; Stöckel et al. 2008; Toepel et al. 2008, 2009). We have demonstrated that gene transcription is highly synchronized during 12 h LD conditions and that the large nitrogenase cluster is coordinately transcribed only in the early part of the dark period. As we varied the light regime to include LL and 6 h LD periods, we were able to discriminate between circadian and diurnal regulation of various genes (Toepel et al. 2009). The nitrogenase and uptake hydrogenase genes are regulated in a circadian fashion and occur only in the dark under 12 or 6 h LD conditions. However, once the cultures have been adapted to LD growth and

then transferred to LL, they are capable of transcribing the nitrogenase genes at a high level (Toepel et al. 2009). On the other hand, the bidirectional hydrogenase typically follows a more diurnal pattern and is transcribed only in the dark when grown under 6 h LD periods.

In addition, there are substantial differences in transcript level between the nitrogenase and uptake hydrogenase on the one hand and the bidirectional hydrogenase on the other hand (Toepel et al. 2008, 2009). Nitrogenase and the uptake hydrogenase genes are usually transcribed in a similar fashion and to very high transcript levels. On the other hand, the bidirectional hydrogenase is always transcribed at quite modest levels in all conditions so far tested and with a peak transcript level that is typically only 1% that of the nitrogenase. On the protein level, we had previously shown that nitrogenase is very prominent during the first few hours of nitrogen fixation in the dark and this was verified by careful proteomic analysis over the 24 h period when grown under 12 h LD cycles. Once again, we could show that peak levels of nitrogenase were far greater than peak levels of the Hox proteins. These are critical factors as we consider overall productivity of hydrogen.

The transcriptomic data for *Cyanothece* 51142 have been published (Stöckel et al. 2008; Toepel et al. 2008, 2009), but the proteomic data are available only now for thorough analysis. The accurate mass and time (AMT) approach has identified 3,616 proteins with high confidence. This includes 70% coverage of gene products from the circular chromosome, but only 48% from the linear chromosome. This is consistent with our transcriptomics results that have indicated little or no expression for many of the genes on the linear chromosome under the conditions so far tested.

This information has been valuable for the overall annotation of the genome, as well as in conjunction with the transcriptomics results to determine if specific proteins are present or not under different growth conditions. Since one of our main objectives is to determine if *Cyanothece* strains are capable of H_2 production, we were particularly interested in protein levels of the three enzymes capable of producing hydrogen: nitrogenase, the uptake hydrogenase, and bidirectional hydrogenase (Table 16.2). The proteomic data are only from cells grown under 12 h LD conditions, but along with previous Western blots can provide a substantial amount of information as to protein levels under different LD regimes. Under nitrogen-fixing conditions, the nitrogenase proteins were the most cyclic proteins in the cell and showed peaks and valleys very similar to that of the gene transcription data – the Nif proteins were present only in the dark. Nonetheless, cells do grow under nitrogen-fixing conditions in LL, and we have shown that the nitrogenase genes are strongly induced in a circadian fashion when grown under LL conditions (Toepel et al. 2008). Additionally, we obtained reasonably high rates of nitrogenase activity in continuous light and we have demonstrated high Nif levels in LL by using Western blots with Nif antibodies (Colon-Lopez et al. 1998). On the other hand, we have shown that the *hupLS* genes are strongly up-regulated in the dark, but are expressed at much lower levels in continuous light (Toepel et al. 2008). Proteomic analysis of the HupL protein indicates that it is resent in high levels in the dark, but only at extremely low levels in the light (Fig. 16.4). This is consistent with our expression

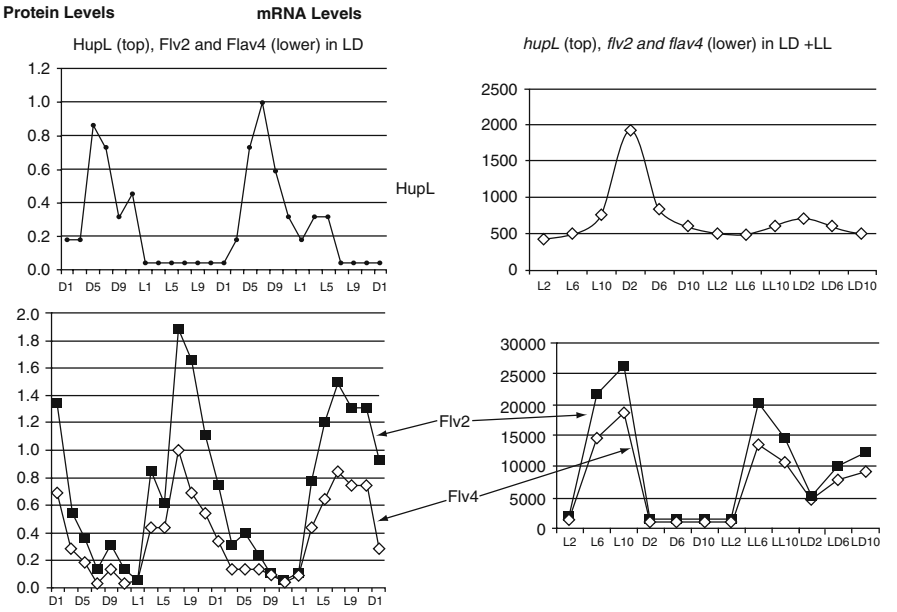


Fig. 16.4 Analysis of protein levels and transcript levels for the HupL proteins, representative of the uptake hydrogenase, and the Flv2 and Flv4 proteins

data which indicated that *hupLS* genes were up-regulated in a circadian fashion in the dark, coincident with the nitrogenase genes (Toepel et al. 2009). Based on these results, it would appear that the HupLS proteins are present, if at all, at very low levels during continuous light. This was the first suggestion that incubating cells under continuous light conditions might be beneficial for hydrogen production. Finally, the *hox* genes were expressed at very low levels in the dark under 12 h LD conditions. When grown under 6 h LD conditions, the *hox* genes were expressed at peaks in the dark but without a substantial trough in the intervening 6 h light period (Toepel et al. 2009). Consistent with the low transcript levels, very low levels of the Hox proteins were identified by proteomics either in the light or in the dark. We are left with the major conclusion that the nitrogenase enzyme is the only hydrogen-producing enzyme present in reasonable quantities during LL growth.

Another type of protein that might be important in permitting hydrogen production is the flavoprotein. Helman et al. (2003) have shown that flavoproteins are essential for photoreduction of O_2 in cyanobacteria via the Mehler reaction. We had determined that an operon containing two flavoproteins was induced under low-O_2 conditions in *Synechocystis* 6803 as well as in *Cyanothece* 51142 (Summerfield, Toepel and Sherman 2008); the data are summarized in Table 16.3. The *flv2* and *flv4* genes are inducible under low-oxygen conditions and we have checked levels of transcript and protein under different light conditions. As shown in Fig. 16.4, the transcript and protein levels of *flv2* and *flv4* were significantly higher in the light than

Table 16.3 Induction of flavoprpteins *flv2* and *flv4* under low O_2 conditions

	Synechocystis genes	Low O_2 induction	*Cyanothece* genes	Low O_2 induction
		t = 1, 2, 6		t = 1, 2, 6
flv1	*sll1521*	1 1 1	cce-2580	1 1 1
flv3	*sll0550*	1 1 1	cce-3635	1 2 1
Flv2	*sll0217*	3 3 3	cce-3835	6 4 2
Flv4	*sll0219*	2 2 2	cce-3833	4 3 2

in the dark, whereas the other two flavoproteins showed little change in expression under these conditions (data not shown). We suggest that these flavoproteins are utilized in the Mehler reaction to reduce oxygen under the appropriate conditions (see Helman et al. 2003). These flavoproteins are in an operon that includes a hypothetical membrane protein with four membrane-spanning regions. Although Flv2 and Flv4 appear to lack ideal $NADP^+$binding sites, the two proteins are likely tethered to the membrane by this membrane protein in such a way as to provide the appropriate activity. This system may help keep the intracellular concentration of oxygen low, thus permitting nitrogenase activity even in the presence of continuous light. These data represented a background as we began our detailed hydrogen production experiments.

16.2.7 Hydrogen Production in Cyanothece *Strains*

We have begun a detailed process of determining hydrogen production in the *Cyanothece* strains under a variety of conditions. Although the most complete work has so far been performed for *Cyanothece* 51142, we have measured hydrogen production under both nitrogen-sufficient and nitrogen-fixing conditions in all of the strains. Importantly, the analysis above led us to incubate N_2-fixing cells in LL under argon to provide a very low-O_2environment. All of the strains produced approximately 2–5 μmol H_2/mg Chl/h when grown under nitrogen-sufficient conditions. We obtained hydrogen production when cells were incubated in the light or dark, but there was an enhancement caused by light incubation. On the other hand, we get significantly more hydrogen produced when cultures are grown under nitrogen-fixing conditions. Strains *Cyanothece* 7424, 7425, 8801, and 8802 all produced ~30–40 μmol H_2/mg Chl/h, whereas *Cyanothece* 7822 and 51142 could produce significantly more. We have not performed as many experiments with *Cyanothece* 7822, but we have consistently obtained extremely high rates (over 150 μmol H_2/mg Chl/h) of hydrogen production with *Cyanothece* 51142 when grown either in the presence or absence of glycerol as a carbon source. The best results are obtained when cells are grown for 3 days under LL and then incubated under argon in LL. A critical feature appears to be the need to protect nitrogenase from oxygen inactivation, and incubation in argon provides that environment. From these results, it

is evident that nitrogenase can produce far more hydrogen than the bidirectional hydrogenase, even when the *hupLS* genes are present. Of course, our previous results suggest that very little HupLS was present under LL incubation. Surprisingly, *Cyanothece* 7425 produced hydrogen at levels no higher than that of many of the other strains that contain an uptake hydrogenase. Thus, the presence or absence of HupLS may not be the most critical factor for H_2 production in all diazotrophic cyanobacteria.

16.3 Discussion

Strains of the cyanobacterial genus *Cyanothece* have become among the best studied of all cyanobacteria. We now have genome sequences for six strains and a detailed proteome for *Cyanothece* 51142. In addition, proteomes for the other sequenced strains are being developed as a way of initiating a process of providing proteomes for many bacterial genomes (D. Koppenaal, personal communication). Proteomics and transcriptomics have provided a great deal of high-throughput data that can be used to analyze metabolic processes in *Cyanothece*. In the current study, we have determined the best conditions under which to measure hydrogen production by *Cyanothece* 51142. At the current time, we have obtained the highest levels of hydrogen in *Cyanothece* 51142 when incubated under argon under LL conditions. This was somewhat surprising, but we believe that it has helped to identify the major metabolic issues concerned with hydrogen production in this strain. It is obvious that nitrogenase is the enzyme that produces the hydrogen and we have also demonstrated that this enzyme is produced continuously under these conditions even though cells do not grow (data not shown). The key energetic features for hydrogen production include plentiful energy (provided by photosynthesis and respiration), reducing power, and protection of the nitrogenase from oxygen (provided by argon and the Mehler reaction mediated by the flavoproteins). We also know from previous results that PS I is highly expressed at the same time as the nitrogenase (Colon-Lopez and Sherman 1998). It is highly likely that PS I is being used both in a cyclic fashion for ATP production and along with the flavoproteins in the Mehler reaction.

Overall, the amount of hydrogen produced by these *Cyanothece* species is quite significant based on previous results with cyanobacteria (Dutta et al. 2005). The high-throughput data have provided background information to help us try various physiological conditions for growth and hydrogen production and such experiments are continuing. These rates have so far been with wild-type strains without any genetic or molecular manipulations as the development of such resources are still in progress. Nonetheless, the initial results are promising and demonstrate the benefit of studying novel photosynthetic microbes as a source of alternative energy sources.

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