Variations in the Rhythms of Respiration and Nitrogen Fixation in Members of the Unicellular Diazotrophic Cyanobacterial Genus *Cyanothece*

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In order to accommodate the physiologically incompatible processes of photosynthesis and nitrogen fixation within the same cell, unicellular nitrogen-fixing cyanobacteria have to maintain a dynamic metabolic profile in the light as well as the dark phase of a diel cycle. The transition from the photosynthetic to the nitrogen-fixing phase is marked by the onset of various biochemical and regulatory responses, which prime the intracellular environment for nitrogenase activity. Cellular respiration plays an important role during this transition, quenching the oxygen generated by photosynthesis and by providing energy necessary for the process. Although the underlying principles of nitrogen fixation predict unicellular nitrogen-fixing cyanobacteria to function in a certain way, significant variations are observed in the diazotrophic behavior of these microbes. In an effort to elucidate the underlying differences and similarities that govern the nitrogen-fixing ability of unicellular diazotrophic cyanobacteria, we analyzed six members of the genus *Cyanothece*. *Cyanothece* sp. ATCC 51142, a member of this genus, has been shown to perform efficient aerobic nitrogen fixation and hydrogen production. Our study revealed significant differences in the patterns of respiration and nitrogen fixation among the *Cyanothece* spp. strains that were grown under identical culture conditions, suggesting that these processes are not solely controlled by cues from the diurnal cycle but that strain-specific intracellular metabolic signals play a major role. Despite these inherent differences, the ability to perform high rates of aerobic nitrogen fixation and hydrogen production appears to be a characteristic of this genus.

Nitrogen fixation is an important global phenomenon by which molecular nitrogen, one of the most abundant components of the earth’s atmosphere, is converted into a more reduced form suitable for incorporation into living systems. The majority of this nitrogen fixation is achieved by biological means through the activity of microorganisms (Burris and Roberts, 1993; Raymond et al., 2004; Rubio and Ludden, 2008). This process is energy intensive, and nitrogenase, the enzyme complex involved in the biological nitrogen fixation reaction, is generally known to be extremely sensitive to oxygen (Robson and Postgate, 1980; Hill et al., 1981; Berman-Frank et al., 2005). Thus, most microbes participating in this process fix nitrogen only when suitable anaerobic or microaerobic conditions are established in an otherwise oxygen-rich environment.

However, some nitrogen-fixing (diazotrophic) microbes have the advantage of being able to fix nitrogen in aerobic environments. Outstanding among these are the photosynthetic prokaryotes called cyanobacteria, an extremely successful group of microbes with plant-like traits. These microbes are considered to be the progenitors of plant chloroplasts. Cyanobacteria perform both oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation, thereby providing a platform to power the most metabolically expensive biological process (Simson and Burris, 1984) with solar energy.

Among the nitrogen-fixing cyanobacteria, filamentous strains have been extensively studied for their contribution to the nitrogen cycle in marine and terrestrial ecosystems (Mulligan and Haselkorn, 1989; Kaneko et al., 2001; Meeks et al., 2001; Sañudo-Wilhelmy et al., 2001; Wong and Meeks, 2001; Gomez et al., 2005). Some of these filamentous strains develop specialized cells called heterocysts that allow the spatial segregation of photosynthesis and nitrogen fixation. These heterocysts also have higher rates of respiratory oxygen consumption, which results in a virtually anoxic environment conducive for the nitrogenase enzyme (Bergman et al., 1997). All heterocystous strains are known to function aerobically. In contrast, nonheterocystous cyanobacteria lack any specialized oxygen-free compartments and often require incubation under microoxic or anaerobic conditions for nitrogen fixation (Rippka and Waterbury, 1977; Rippka et al., 1977; Brass et al., 1992). However, some nonheterocystous cyanobacterial strains can fix nitrogen under aerobic conditions. These include...
some filamentous genera like *Trichodesmium* spp., *Lyngbya* spp., and *Oscillatoria* spp. (Jones, 1990; Janson et al., 1994; Finzi-Hart et al., 2009) as well as unicellular genera like *Gloeothecae* spp. and *Cyanothece* spp. (Wyatt and Silvey, 1969; Rippka and Waterbury, 1977; Huang and Chow, 1988; Van Ni et al., 1988; Schütz et al., 2004).

In comparison with filamentous cyanobacteria, which have long been recognized for their nitrogen-fixing ability, the importance of unicellular cyanobacteria as key components of the environmental nitrogen cycle has only been recently uncovered. Studies over the last decade have established unicellular strains like *Crocosphaera* spp., *Cyanothece* spp., and UCYN-A as important players in the marine nitrogen cycle (Zehr et al., 2001; Montoya et al., 2004; Zehr, 2011). Since unicellular diazotrophic cyanobacteria utilize the same cellular platform for photosynthesis and nitrogen fixation, they are required to adjust their cellular metabolism to accommodate these two antagonistic processes. Systems-level studies in the unicellular genus *Cyanothece* have revealed a temporal separation of the two processes, photosynthesis occurring during the day and nitrogen fixation occurring at night (Stöckel et al., 2008; Toepel et al., 2008; Welsh et al., 2008). Cellular respiration plays a critical role during the transition from one phase to the next, rapidly freeing the intracellular environment of the photosynthetically generated oxygen and rendering it conducive for the induction of nitrogenase activity. In addition, respiration also sustains the process of nitrogen fixation, not only by maintaining a low-oxygen environment required for the functioning of the nitrogenase enzyme but also by mobilizing the stored solar energy to fuel this energy-intensive process.

Unicellular diazotrophs exhibit great diversity in the efficiency of nitrogen fixation as well as in the physiological regulation of the process. For instance, members of the genus *Gloeothecae* fix nitrogen aerobically during the day, but at 0% dissolved oxygen concentration, nitrogen fixation is shifted entirely to the dark period (Ortega-Calvo and Stal, 1991; Taniuchi et al., 2008). In contrast, some *Synechococcus* spp. strains can fix nitrogen only when incubated under anoxic conditions (Steunou et al., 2006). Members of the genus *Cyanothece* have been reported to engage in both aerobic and anaerobic nitrogen fixation, with nitrogenase activity peaking during the night (Reddy et al., 1993; Bergman et al., 1997; Turner et al., 2001). This suggests that, in addition to the regulations imposed by the diurnal cycle, strain-specific intracellular cues govern the process of nitrogen fixation in unicellular cyanobacteria, which may vary according to the genotype or the ecotype of the strains.

Members of the unicellular cyanobacterial genus *Cyanothece* are diazotrophs that thrive in marine as well as terrestrial environments. This genus was originally grouped together with *Synechococcus* spp. but was later separated on the basis of distinct morphological and biochemical differences between the two genera (Komárek, 1976; Rippka and Cohen-Bazire, 1983). Some of the features that define the largely heterogeneous genus *Cyanothece* are oval to cylindrical cells, larger than 3 μm in size (they can be as large as 24 μm in diameter), radially arranged thylakoids, and a mucilaginous layer surrounding the cells (Komárek and Cepák, 1998; Porta et al., 2000; Liberton et al., 2011).

It was recently demonstrated that *Cyanothece* sp. ATCC 51142, a member of the genus *Cyanothece*, has the unique ability to produce molecular hydrogen at exceptionally high rates under aerobic conditions (Bandyopadhyay et al., 2010). This striking observation was attributed to the nitrogenase enzyme system of *Cyanothece* sp. ATCC 51142. Our study also indicated that high rates of respiration in this strain might contribute to its nitrogenase-mediated aerobic hydrogen production. Glycerol was found to be an efficient source of reductants and energy for this process. In an effort to investigate if this atypical cyanobacterial trait was a characteristic of the genus *Cyanothece*, five additional *Cyanothece* spp. strains from different ecological habitats were sequenced to completion. The six strains display more than 90% identity in their 16S ribosomal RNA sequence but exhibit striking variability with respect to their genome sizes (with the largest genome being 7.8 Mb and the smallest being 4.4 Mb), the number of plasmids, and the percentage of pseudogenes (Bandyopadhyay et al., 2011). In addition, two of the strains possess linear chromosomal elements, features not known to occur in any other photosynthetic bacteria sequenced to date, which may impart niche-specific advantages to these strains. Analysis of the genome sequence of the *Cyanothece* sp. strains showed the presence of a nitrogenase gene cluster in all five strains, and preliminary analysis showed that four of the five strains were capable of aerobic nitrogen fixation and hydrogen production (Bandyopadhyay et al., 2011). In this study, we have focused on the patterns of nitrogen fixation and respiration in six different *Cyanothece* spp. strains in an effort to elucidate the underlying differences and similarities in these processes in unicellular diazotrophic strains with similar genotypic but varied ecological backgrounds. Our study reveals inherent differences in the regulation of these processes, which are likely controlled by strain-specific cellular signals. However, despite the differences in the patterns of nitrogenase activity, aerobic nitrogen fixation and hydrogen production was found to be a characteristic of this genus, with most members exhibiting nitrogenase-mediated hydrogen production at rates higher than any other wild-type cyanobacterial strain.

**RESULTS**

*Cyanothece* spp. Strains Exhibit Diverse Phenotypes

The six *Cyanothece* spp. strains isolated from different geographical regions exhibit heterogeneity with
respect to cell size, shape, and color (Fig. 1). All six strains have cells larger than 3 \( \mu m \) (Fig. 2), and all but \textit{Cyanothece} sp. PCC 7425 have regions of radially arranged thylakoid membranes, consistent with the definitions of the genus \textit{Cyanothece} (Komárek, 1976; Rippka and Cohen-Bazire, 1983). \textit{Cyanothece} sp. PCC 7822 has the largest cells (8–10 \( \mu m \)), followed by \textit{Cyanothece} sp. PCC 7424 (6–8 \( \mu m \)). \textit{Cyanothece} sp. PCC 7425 cells are the smallest in size (3–4 \( \mu m \)) and are more coccoid in shape compared with the oval shape of the other \textit{Cyanothece} spp. cells. At the ultrastructural level, the \textit{Cyanothece} spp. strains differ in the thylakoid membrane architecture as well as in the type of inclusion bodies (Fig. 2). Three strains isolated from terrestrial ecotypes (\textit{Cyanothece} sp. PCC 8801, PCC 7424, and PCC 7822) possess the pigment phycoerythrin, which imparts a brownish green color to the cells (Fig. 1, A and B).

**Figure 1.** Phenotypic variations among members of the genus \textit{Cyanothece}. A, Cultures of the six \textit{Cyanothece} spp. strains showing differences in pigment composition. B, Absorption spectra of the six \textit{Cyanothece} spp. strains showing the phycoerythrin peak (green arrow) in three of the six strains. C, Light micrographs of the six \textit{Cyanothece} spp. strains showing differences in size, shape, and color of the cells. a, \textit{Cyanothece} 51142, b, \textit{Cyanothece} sp. PCC 7424, c, \textit{Cyanothece} sp. PCC 7425, d, \textit{Cyanothece} sp. PCC 7822, e, \textit{Cyanothece} sp. PCC 8801, f, \textit{Cyanothece} sp. PCC 8802. The strains were grown in the presence of combined nitrogen sources in the medium and analyzed in the log phase of growth (between 5 and 6 d after inoculation). Bar = 5 \( \mu m \).
is a marine strain, whereas the other *Cyanothece* spp. strains have been isolated from rice fields. The strains exhibit different growth characteristics under azotrophic and diazotrophic conditions (Fig. 3). *Cyanothece* sp. PCC 7424 and PCC 7822 cells have a propensity to form clumps, and these two strains grow slower compared with the other strains both in the presence and absence of combined sources of nitrogen in the medium (Fig. 3A). The strains exhibit a longer lag phase under nitrogen-fixing conditions, and the length of the lag phase varies among the strains. Of the six strains, *Cyanothece* sp. ATCC 51142 appears to be the most efficient in fixing atmospheric nitrogen, followed by *Cyanothece* sp. PCC 8802, growing rapidly under nitrogen-fixing conditions (Fig. 3B). *Cyanothece* sp. PCC 7424, PCC 7822, and PCC 8801 also grow in medium lacking sources of fixed nitrogen, but they exhibit much reduced doubling time. In contrast, *Cyanothece* sp. PCC 7425 cells are not as efficient in fixing atmospheric nitrogen, as is evident from the cultures turning yellow within a few days of growth in nitrogen-deficient medium (Fig. 3B).

**Comparative Analysis of Nitrogen Fixation and Hydrogen Production in the *Cyanothece* spp. Strains**

Earlier studies have indicated that the members of the genus *Cyanothece* can engage in either aerobic or anaerobic nitrogen fixation (Waterbury and Rippka, 1989; Turner et al., 2001; Bandyopadhyay et al., 2011). Two of the five *Cyanothece* spp. strains (PCC 7424 and PCC 7425) included in this study were categorized as anaerobic nitrogen-fixing strains (Bergman et al., 1997). We studied the six *Cyanothece* spp. strains for their ability to fix nitrogen and produce hydrogen under various growth and incubation conditions. Under all tested conditions, *Cyanothece* sp. ATCC 51142 exhibited the highest rates of nitrogenase activity and hydrogen production. The nitrogenase activity of the strains was measured in terms of acetylene reduction and expressed as ethylene production (Oda et al., 2005). Ethylene or hydrogen production in *Cyanothece* sp. ATCC 51142 cells grown under photoautotrophic conditions and incubated in air was about 150 μmol mg⁻¹ chlorophyll h⁻¹ (Fig. 4; Tables I and II). Under similar conditions, *Cyanothece* sp. PCC 8802 fixed nitrogen at approximately 100 μmol ethylene mg⁻¹ chlorophyll h⁻¹ and produced hydrogen at approximately 50 μmol mg⁻¹ chlorophyll h⁻¹. The lowest nitrogenase activity (approximately 50 μmol mg⁻¹ chlorophyll h⁻¹) was observed in *Cyanothece* sp. PCC 7424 and PCC 7822, and the hydrogen production rate in these two strains was also about 50 μmol mg⁻¹ chlorophyll h⁻¹. *Cyanothece* sp. PCC 8801 showed intermediate rates of nitrogenase activity (approximately 75 μmol ethylene mg⁻¹ chlorophyll h⁻¹), while hydrogen production was approximately 40 μmol mg⁻¹ chlorophyll h⁻¹. In *Cyanothece* sp. PCC 7425 cells incubated under aerobic conditions, no nitrogenase activity or hydrogen production could be detected.

When cells grown under photoautotrophic conditions were incubated in an anoxic environment, the specific rates of nitrogen fixation and hydrogen production were significantly enhanced. Under anaerobic incubation, the rate of nitrogenase activity in *Cyanothece* sp. ATCC 51142 cells was more than 230 μmol mg⁻¹ chlorophyll h⁻¹, and compared with this, the rate of hydrogen production was significantly higher (approximately 350 μmol mg⁻¹ chlorophyll h⁻¹). In *Cyanothece* sp. PCC 7425 incubated under anoxic conditions, the rate of ethylene and hydrogen production was about 40 μmol mg⁻¹ chlorophyll h⁻¹. In all other *Cyanothece* spp. strains, the specific rate of anaerobic nitrogen fixation and hydrogen production varied between 150 and 200 μmol mg⁻¹ chlorophyll h⁻¹, with hydrogen production rates being higher than or equal to the rates of ethylene production.

Photomixotrophic growth in the presence of glycerol was shown to significantly enhance the rate of hydrogen production in *Cyanothece* sp. ATCC 51142 (Bandyopadhyay et al., 2010). We tested the effect of glycerol on nitrogen fixation and hydrogen production in the other *Cyanothece* spp. strains. Unlike *Cyanothece*

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**Figure 2.** Electron micrographs of *Cyanothece* spp. strains. A, *Cyanothece* sp. ATCC 51142; B, *Cyanothece* sp. PCC 7424; C, *Cyanothece* sp. PCC 7425; D, *Cyanothece* sp. PCC 7822; E, *Cyanothece* sp. PCC 8801; F, *Cyanothece* sp. PCC 8802. All strains were harvested after 5 to 6 d of growth and prepared for electron microscopy by high-pressure freezing. Intracellular features are labeled: C, carboxysomes; G, glycogen granules; P, polyphosphate bodies; T, thylakoid membranes. Bars = 500 nm.
sp. ATCC 51142, which utilizes glycerol for the production of biomass (Feng et al., 2010), the other Cyanothece spp. strains did not exhibit any significant difference in growth rate when grown in the presence of glycerol (data not shown). However, some of the Cyanothece spp. strains could use glycerol for nitrogen fixation and hydrogen production (Tables I and II). In Cyanothece sp. ATCC 51142, the cells supplemented with glycerol fixed nitrogen at more than 200 μmol ethylene mg⁻¹ chlorophyll h⁻¹ and produced hydrogen at more than 250 μmol mg⁻¹ chlorophyll h⁻¹ when incubated under aerobic conditions. Under anaerobic incubation conditions, these rates were approximately 300 and 450 μmol mg⁻¹ chlorophyll h⁻¹, respectively. In Cyanothece sp. PCC 7425, glycerol enhanced the rates of nitrogen fixation and hydrogen production (approximately 60 μmol ethylene mg⁻¹ chlorophyll h⁻¹) and hydrogen production (approximately 80 μmol mg⁻¹ chlorophyll h⁻¹) by about 1.5- to 2-fold. In Cyanothece sp. PCC 7424 and PCC 7822, glycerol also contributed toward higher nitrogen fixation and hydrogen production rates (Tables I and II). In contrast, glycerol had an adverse effect on nitrogen fixation in Cyanothece sp. PCC 8801 and PCC 8802, although hydrogen production remained unchanged.

Phase Differences in the Diurnal Cycling of Nitrogenase Activity in the Cyanothece spp. Strains

We assessed the diurnal rhythms in nitrogenase activity in five of the six Cyanothece spp. strains where nitrogenase activity could be measured under aerobic incubation conditions. Cells grown under diazotrophic conditions and subjected to 12-h light/dark cycles were sampled every 2 h over a period of 3 d for nitrogenase activity analysis. The six strains differed considerably with respect to the time point in the diurnal cycle when nitrogenase activity was induced, the period for which the activity lasted, as well as the specific activity of the nitrogenase enzyme (Fig. 5). In Cyanothece sp. ATCC 51142, nitrogenase activity was induced toward the later half of the light phase, between L6 and L10, and the activity peaked in the early phase of the dark cycle (between D2 and D6). In contrast, nitrogenase activity in the other four Cyanothece spp. strains was induced at the beginning or the middle of the dark phase and peaked between D6 and D12. The period for which the nitrogenase activity could be detected was longest in Cyanothece sp. ATCC 51142, and it could last up to 12 h. In Cyanothece sp. PCC 8801 and PCC 8802, the activity lasted between 8 and 10 h. Cyanothece sp. PCC 7424 and PCC 7822 showed delayed nitrogenase induction, with activity being detected at D6. The activity lasted for 6 h in these two strains. While in Cyanothece sp. ATCC 51142, no nitrogenase activity could be detected after the end of the dark cycle, in the other Cyanothece spp. strains, nitrogenase activity extended into the early phase of the light cycle (L2). Of the five strains, the highest nitrogenase activity was observed in Cyanothece sp. ATCC 51142, followed by Cyanothece sp. PCC 8802, PCC 8801, and PCC 7822 (Fig. 5).

Temporal Synchronization of Respiration and Nitrogenase Activities in the Cyanothece spp. Strains

We studied the respiratory and nitrogenase activities in the different Cyanothece spp. strains grown under 12-h light/dark cycles. The dissolved oxygen level in the cultures reflected the respiratory activities of the cells, whereas nitrogenase activity was determined by measuring the rate of acetylene-to-ethylene conversion. Our observations revealed significant differences in the respiratory and nitrogen fixation activities in the studied strains (Fig. 6). Interestingly, the fluctuations in the dissolved oxygen level, corresponding to the photosynthetic and respiratory activities of the cells, varied considerably in the six Cyanothece spp. cultures that were grown under identical conditions. The rate

Figure 3. Growth characteristics of the six Cyanothece spp. strains. Growth curves are shown for Cyanothece spp. strains grown in the presence of combined sources of nitrogen (A) and in the absence of combined nitrogen sources (B). The diazotrophic cultures were started as described in “Materials and Methods,” and the diazotrophic nature of the cells was ensured by assessing their nitrogen-fixing ability. Blue, Cyanothece sp. ATCC 51142; orange, Cyanothece sp. PCC 8802; purple, Cyanothece sp. PCC 8801; green, Cyanothece sp. PCC 7424; brown, Cyanothece sp. PCC 7822; red, Cyanothece sp. PCC 7425.
of respiratory oxygen uptake was significantly different in the strains, as revealed by the differences in the slopes. The dissolved oxygen level dropped close to 0 μM at the peak of dark respiration in some cultures, while in others it dropped to only 50% of the concentration achieved at the peak of photosynthesis. The time for which the minimum oxygen level was maintained in each of the strains during the dark period also varied. This was apparent in the width of the valleys at the corresponding time points. In *Cyanobacterium* sp. ATCC 51142, a sharp rise in the oxygen level was observed at the beginning of the light cycle, and the highest oxygen concentration corresponding to the peak photosynthetic activity was attained within the first few hours of the dark period (Fig. 6A). Once the peak was attained, a rapid 10% drop in the oxygen level was observed by the middle of the day. This oxygen concentration was maintained until the beginning of the dark period, when another sharp drop was observed, bringing the dissolved oxygen to about 40% of the peak level. The oxygen concentration was maintained at this level (approximately 175 μM) for the entire dark period.

In contrast to *Cyanobacterium* sp. ATCC 51142, *Cyanobacterium* sp. PCC 7424, PCC 7822, PCC 8801, and PCC 8802 cultures exhibited an increase in the dissolved oxygen level for almost the entire light period, and the concentration started to drop only at the onset of the dark period (Fig. 6, B, D, and E). Also in contrast to *Cyanobacterium* sp. ATCC 51142, the drop in the dissolved oxygen level was gradual in these strains, decreasing over the entire dark period. The oxygen concentration at the end of the dark period was close to 0 μM in *Cyanobacterium* sp. PCC 7424, PCC 7822, and PCC 8802. In *Cyanobacterium* sp. PCC 8801, a shift was observed in the dissolved oxygen concentration over the 72-h assay period in the bioreactor, with the lowest concentration recorded at mid dark, late dark, and early light phases of the diel cycles. In this strain, the lowest oxygen concentration attained at the peak of respiration was 50 μM. In *Cyanobacterium* sp. PCC 7424, PCC 7822, and PCC 8801, the minimum dissolved oxygen concentration was maintained for only a brief period, whereas in *Cyanobacterium* sp. PCC 8802, a pattern similar to *Cyanobacterium* sp. ATCC 51142 was observed, where the minimum oxygen levels were maintained for several hours. The respiration in *Cyanobacterium* sp. PCC 7425 was distinct from the other *Cyanobacterium* spp. strains, with the dissolved oxygen levels decreasing slowly over the entire dark period to about 30% of the initial level and then increasing gradually over the entire light period. While to some extent this resembles the pattern observed in *Cyanobacterium* sp. ATCC 51142, unlike *Cyanobacterium* sp. ATCC 51142, the oxygen level in *Cyanobacterium* sp. PCC 7425 does not stabilize at the lowest concentration and shows an immediate rise upon reaching the lowest point. These results demonstrate that respiration and photosynthesis are regulated differently in the six *Cyanobacterium* spp. strains subjected to identical external cues, suggesting differences in the metabolic status of the cells, which translates into dissimilar regulatory signals.

In parallel, we assayed nitrogenase activity in the six *Cyanobacterium* spp. cultures, sampling every 2 h for a period of 3 d. The highest rate of nitrogen fixation was observed in *Cyanobacterium* sp. ATCC 51142 (120 μmol mg⁻¹ chlorophyll h⁻¹). In this strain, nitrogenase activity induction coincided with the dip in dissolved oxygen concentration before the end of the light period, reached its peak by the first few hours of the dark period, and diminished prior to the end of the dark period (Fig. 6A). In *Cyanobacterium* sp. PCC 7424, the nitrogenase activity is induced in the middle of the dark cycle and reaches a peak toward the end of the dark
period, coinciding with the respiratory peak. The activity extends into the next light cycle and subsides when the oxygen concentration increases. In *Cyanothece* sp. PCC 7822, the nitrogenase activity is detected toward the last few hours of the dark period and also extends a couple of hours into the next light cycle. Thus, in both *Cyanothece* sp. PCC 7424 and PCC 7822, the activity period of the nitrogenase enzyme is short, lasting between 6 and 8 h. In *Cyanothece* sp. PCC 8801 and PCC 8802, nitrogenase activity is induced between early dark (D2) and late dark phase, depending on the dissolved oxygen concentration. Although the peak activity subsides before the end of the dark period, low levels of activity can be detected even in the middle of the light period in these two strains. In *Cyanothece* sp. PCC 7425, no nitrogenase activity could be measured under the above assay conditions.

### Chromosomal Clustering of *nitrogenase* Genes in Unicellular, Aerobic Nitrogen-Fixing Cyanobacteria

To determine if the ability to perform aerobic nitrogen fixation and hydrogen production is conferred by any unique feature in the *nitrogenase* (*nif*) gene cluster in the members of the genus *Cyanothece*, we examined the level of conservation of the entire chromosomal cluster containing the *nif* genes. The analysis included 12 sequenced unicellular nitrogen-fixing cyanobacterial strains: *Cyanothece* sp. ATCC 51142, CCY 0110, PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802, *Crocosphaera* sp. WH 8501, the uncultured cyanobacterium UCYN-A, and three anaerobic nitrogen-fixing *Synechococcus* spp. strains. We used *Cyanothece* sp. ATCC 51142, the strain with the largest contiguous *nif* gene cluster (Welsh et al., 2008; Bandyopadhyay et al., 2011) and with the highest nitrogen-fixing and hydrogen-producing ability, as the reference strain and compared its sequence with the other 11 strains. We selected a 28.3-kb contiguous region located between positions 555,439 and 583,778 in the genome of *Cyanothece* sp. ATCC 51142. This region consists of 35 protein-coding genes (cce_0545–cce_0579), which includes most of the known *nif* genes. Genes are clustered in two groups, oriented in opposite directions, as shown in Figure 7.

The marine *Cyanothece* strain sp. CCY 0110 shows the highest level of homology to *Cyanothece* sp. ATCC 51142, with around 90% identify at nucleotide level, and contains almost the entire reference sequence (except 171 bp; colored in black in Fig. 7) as a contiguous region in its genome. *Crocosphaera* sp. WH 8501, another marine strain, shows the next highest level of conservation, with about 82% overall identify in the aligned regions. However, no homologous regions corresponding to the sequences of protein-coding genes cce_0551, cce_0556, cce_0558, and cce_0577 are found within this genome.

*Cyanothece* sp. PCC 8801 and PCC 8802 showed a very similar pattern of alignment, with about 80% and

### Table 1. Effect of exogenous carbon source on nitrogenase activity in *Cyanothece* spp.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Photoautotrophic</th>
<th>Photomixotrophic</th>
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<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td></td>
<td>μmol ethylene produced mg⁻¹ chlorophyll h⁻¹</td>
<td></td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. ATCC 51142</td>
<td>148.45 ± 13.4</td>
<td>238.2 ± 46.4</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 7424</td>
<td>55.3 ± 12.3</td>
<td>141.0 ± 32.2</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 7425</td>
<td>50.5 ± 7.8</td>
<td>36.7 ± 7.8</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 7822</td>
<td>48.83 ± 12.5</td>
<td>130 ± 26.4</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 8801</td>
<td>89.2 ± 10.2</td>
<td>162.2 ± 21.4</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 8802</td>
<td>98.2 ± 10.6</td>
<td>173.6 ± 36.2</td>
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</tbody>
</table>

Error is reported as SD from the average. Photoautotrophic indicates growth in the presence of light and ambient CO₂, and photomixotrophic indicates growth in the presence of light and 50 mM glycerol.

### Table 2. Effect of exogenous carbon source on hydrogen production in *Cyanothece* spp.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation</th>
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<tbody>
<tr>
<td></td>
<td>Photoautotrophic</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td></td>
<td>μmol hydrogen mg⁻¹ chlorophyll h⁻¹</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. ATCC 51142</td>
<td>133.21 ± 21.5</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 7424</td>
<td>50.7 ± 19.3</td>
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<td><em>Cyanothece</em> sp. PCC 7425</td>
<td>0</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 7822</td>
<td>50.5 ± 9.6</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 8801</td>
<td>53.3 ± 11.2</td>
</tr>
<tr>
<td><em>Cyanothece</em> sp. PCC 8802</td>
<td>61.2 ± 10.8</td>
</tr>
</tbody>
</table>

Error is reported as SD from the average. Photoautotrophic indicates growth in the presence of light and ambient CO₂, and photomixotrophic indicates growth in the presence of light and 50 mM glycerol.
Nitrogen Fixation in Unicellular Cyanobacteria

Figure 5. Variations in the diurnal activities of the nitrogenase enzyme in the six Cyanothece spp. strains grown under 12-h light/dark cycles. The assay was performed with cells in the logarithmic phase of growth (6- to 7-d-old culture). The optical density of the cultures were measured each day and adjusted to ensure similar cell density throughout the assay.

DISCUSSION

Unicellular nitrogen-fixing cyanobacteria exhibit great diversity in their diazotrophic behavior, particularly in the efficiency of nitrogen fixation, the induction of nitrogenase activity, the phase of the diurnal cycle when the nitrogenase activity peaks, and in their ability to perform aerobic versus anaerobic nitrogen fixation. These differences can be attributed to the genotypic constitution of the strains as well as to the selective pressures imposed on them in a particular habitat. Our analysis of the six morphologically and metabolically distinct members of the genus Cyanothece revealed that the extent of these variations is considerable even at an intragenus level. However, despite these variations, high rates of aerobic nitrogen fixation and hydrogen production appear to be a characteristic of this genus, with five of the six strains exhibiting aerobic hydrogen production at rates higher than any other known wild-type cyanobacterial strain (Dutta et al., 2005). Cyanothece sp. PCC 7425 was the only exception in which no nitrogenase activity or hydrogen production could be detected unless an anaerobic incubation environment was provided. These observations corroborate earlier studies, which indicated that Cyanothece sp. PCC 7425 is unique among the Cyanothece spp. strains (Porta et al., 2000; Bandyopadhyay et al., 2011) and in some respects resembles the anaerobic nitrogen-fixing Synechococcus spp. strains. Comparative analysis studies, however, revealed significant differences between Cyanothece sp. PCC 7425 and the anaerobic nitrogen-fixing Synechococcus spp. strains at the genome level and showed its proximity to A. marina in the phylogenetic tree (Bandyopadhyay et al., 2011). Thus, Cyanothece sp. PCC 7425 appears to be an independently evolving strain that might be considered for status outside the Cyanothece genus.

75% of coverage of the reference sequence, respectively. The uncultured marine cyanobacterium UCYN-A, a strain that has undergone drastic genome reduction, has maintained its nif gene cluster, which shares about 65.2% homology with the reference sequence. Cyanothece sp. PCC 7424 and PCC 7822 exhibit about 65% and 60% homology with the reference sequence, respectively. Our analysis revealed that some of the Cyanothece sp. ATCC 51142 nif cluster regions (represented by hatched lines) are present at distant locations in the genomes of Cyanothece sp. PCC 7424, PCC 7822, PCC 8801, and PCC 8802 (Fig. 7), suggesting possible rearrangement of the genomes during evolution.

Among the six Cyanothece spp. strains, Cyanothece sp. PCC 7425 appears to have the most divergent nif gene cluster, sharing only 42% identity with the reference sequence. The nif cluster in this strain is split into two large segments, which are located at distant positions in the genome separated by a fragment of more than 2.5 Mb. At the chromosomal level, large segments of the reference cluster are found to be missing in Cyanothece sp. PCC 7425, as represented by the black segments in Figure 7. Although most of the nif genes that are present in the aerobic nitrogen-fixing strains are present somewhere in the Cyanothece sp. PCC 7425 genome, the integrity of the gene neighborhoods in the nif cluster is destroyed, suggesting that regulatory functions might be impaired. The three anaerobic nitrogen-fixing Synechococcus spp. strains included in this analysis showed Cyanothece sp. PCC 7425-like alignment with the reference strain, exhibiting less than 45% homology with the Cyanothece sp. ATCC 51142 nif cluster. Although parts of the cluster in all four strains showed about 60% to 80% identity at the nucleotide level with the other unicellular diazotrophic strains included in this analysis, more than 50% of the cluster did not exhibit any similarity at the nucleotide level. We generated a phylogenetic tree using the nifH protein sequence from 103 cyanobacterial strains with the Cyanothece sp. ATCC 51142 nifH sequence as the reference (Supplemental Fig. S1). All the aerobic nitrogen-fixing strains branched off from the same node as Crocosphaera watsonii and UCYN-A and clustered together. In contrast, Cyanothece sp. PCC 7425 appears to have branched independently from a point that also gave rise to some of the anaerobic nitrogen-fixing filamentous Oscillatoria spp. strains. The anaerobic nitrogen-fixing Synechococcus spp. strains appear to branch separately from Cyanothece sp. PCC 7425. This analysis revealed that the nif clusters in unicellular marine strains Cyanothece sp. ATCC 51142 and CCY 0110 and Crocosphaera sp. WH 8501 closely resemble each other in the synteny of the genes as well as in the conservation of the nucleotide sequence of the cluster. The nif cluster in anaerobic unicellular nitrogen-fixing strains, on the other hand, holds features distinct from the marine strains, which suggests an independent line of evolution.
The specific activity of the nitrogenase enzyme varied in the six *Cyanothece* spp. strains, resulting in different rates of nitrogen fixation and hydrogen production. The ratio of nitrogenase activity to hydrogen production under aerobic and anaerobic conditions also varied among the six strains (Fig. 4). These observations could be attributed to differences in the efficiency of the nitrogenase and the uptake hydrogenase enzymes, disparity in the oxygen tolerance levels of the nitrogenase enzymes, as well as the contribution of the bidirectional hydrogenase enzyme system toward hydrogen production. In addition, the *nif* clusters differed significantly among the *Cyanothece* spp. strains, a factor that might also contribute to the observed physiological differences. A comparison of this cluster among unicellular cyanobacteria revealed a distinct configuration in the anaerobic nitrogen-fixing strains, with a significant reduction in cluster size (Fig. 7), indicating the possibility that certain segments of the cluster are necessary for aerobic nitrogen fixation. Another interesting observation was the resemblance of the *nif* cluster in the marine strains with *Cyanothece* spp. ATCC 51142 and CCY 0110 and *Crocosphaera* spp. exhibiting more than 90% identity at the nucleotide level. Studies focusing on the nitrogen-fixing abilities of these three strains might lead to important conclusions about the significance of this cluster in aerobic nitrogen fixation and hydrogen production.

Although none of the *Cyanothece* spp. genomes appear to harbor genes for a glycerol uptake facilitator (GlpF), most of the *Cyanothece* spp. strains are capable of utilizing glycerol, suggesting that glycerol transport...
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Their respiratory rates to scavenge excess oxygen as a strategy to protect the nitrogenase enzyme. It was shown that in _Crocospheera_ spp., dark respiration-mediated quenching of the intracellular oxygen concentration was a prerequisite for the induction of nitrogenase activity (Compaoré and Stal, 2010). Respiration also generates the energy essential for the nitrogen fixation process, necessitating the maintenance of a steady rate of respiration during the nitrogen fixation period (Fay, 1992). Studies at the transcriptional and translational levels in _Cyanotheca_ sp. ATCC 51142 have shown that genes involved in respiration and nitrogen fixation are coregulated, with both categories of genes being up-regulated throughout the entire dark period and down-regulated throughout the light period (Stöckel et al., 2008).

Our study revealed a close association between the respiratory and nitrogen-fixing activities of the _Cyanotheca_ spp. strains. The concentration of dissolved oxygen in the growth medium showed strong oscillations over a diurnal cycle, and the pattern of oscillations varied among the _Cyanotheca_ spp. strains. These oscillations in the dissolved oxygen concentration in the medium mainly arise from photosynthetic activities of the cells (which result in a daytime oxygen peak) as well as from dark respiration (which results in the valleys). In addition, photorespiration and the Mehler reaction are known to contribute to the net oxygen concentration of the cultures (Kana, 1992). In most of the _Cyanotheca_ spp. strains, respiratory activities commenced at the beginning of the dark period and peaked toward the end, when a peak in nitrogenase activity was also observed. _Cyanotheca_ sp. ATCC 51142 was an exception, where very high rates of photosynthesis and an early photosynthetic peak were observed in the light period followed by a drop in the dissolved oxygen concentration. While this decrease in oxygen concentration during the light period may suggest a slowing down or cessation of photosynthetic activities, it might also indicate a high basal rate of light-independent respiration and simultaneous consumption of oxygen by processes like photorespiration and the Mehler reaction. A high rate of respiration (considered high for cyanobacteria), which increases under dark cycles, has been reported for _Cyanotheca_ sp. ATCC 51142 (Schneegurt et al., 1997). Light-dependent oxygen consumption was mainly attributed to the Mehler reaction in _Trichodesmium_ spp. (Kana, 1992). As the oxygen concentration in _Cyanotheca_ sp. ATCC 51142 dropped, an early onset of nitrogenase activity was observed (Fig. 6). In contrast, _Cyanotheca_ sp. PCC 7424 and PCC 7822 strains showed a drop in the dissolved oxygen concentration, starting only at the end of the dark period, while _Cyanotheca_ sp. PCC 7425 and PCC 8802 showed a decline only after a couple of hours into the light period. This delay in the onset of respiratory activities might indicate a slower initial respiration rate in these strains, which picks up at L2 in _Cyanotheca_ sp. PCC 8802, leading to a precipitous drop. In _Cyanotheca_ sp. PCC 7425, the drop in oxygen concentration is more gradual and lasts throughout the
dark period. Unlike the other *Cyanothece* spp. strains, the oxygen cycling in *Cyanothece* sp. PCC 8801 showed a phase shift. In the first diurnal cycle, the oxygen concentration started to increase in the middle of the dark period. In the next diurnal period, the rise in oxygen concentration was observed after L9, whereas in the third cycle, the concentration increased only in the light period. This initial anomaly in the cycling pattern is difficult to interpret and might suggest an early cessation of respiration and consequently a rise in the dissolved atmospheric oxygen. It also indicates that there might be a lag before the circadian and diurnal clocks are in sync with the metabolism in *Cyanothece* sp. PCC 8801 under the bioreactor conditions.

While most strains showed high rates of respiration during the dark period, in some strains, the dissolved oxygen level reached close to zero at the peak of respiration. However, surprisingly, these strains did not exhibit high nitrogenase activity. In contrast, *Cyanothece* sp. ATCC 51142 showed very high initial rates of respiration but maintained a higher dissolved oxygen level throughout the dark period, and the nitrogenase activity in this strain was the highest. Thus, maintaining the oxygen concentration at a certain threshold for an extended period of time appears to be a factor that contributes to high rates of nitrogenase activity in *Cyanothece* sp. ATCC 51142. A steady rate of respiration throughout the dark period indicates sufficient availability of reductants and energy in this strain. These observations also suggest that the oxygen tolerance levels of the nitrogenase enzymes in the *Cyanothece* spp. strains might be different, with some strains requiring a more anaerobic environment for the induction of nitrogenase activity. In *Cyanothece* sp. PCC 7425, the slower rate of respiration and high partial pressure of dissolved oxygen appear to be impediments in its ability to fix nitrogen aerobically and necessitate nitrogenase induction by artificial anaerobic conditions. Some components of the *nif* cluster that are missing in *Cyanothece* sp. PCC 7425 and are present in the aerobic nitrogen-fixing strains might also play a crucial role in aerobic nitrogen fixation. Examples of these include the gene encoding the 4Fe-4S ferredoxin iron-sulfur binding domain protein located between *nifB* and *nifS* in the aerobic nitrogen-fixing strains as well as other hypothetical genes in the cluster. In addition, *cysE2*, the first gene in one of the two transcriptional units in this cluster in *Cyanothece* sp. ATCC 51142, is not present in any of the *nif* clusters in *Cyanothece* sp. PCC 7425. Interestingly, coexpression network analysis studies revealed a strong temporal connectivity between *cysE2* and *nifB*, the first gene in the second transcription unit of this cluster in *Cyanothece* sp. ATCC 51142. The location of these two genes is conserved in all the aerobic nitrogen-fixing unicellular cyanobacterial strains, and a temporal connectivity between these genes might implicate their role in initiating aerobic nitrogen fixation. The period for which the nitrogenase activity lasted also varied in the *Cyanothece* spp. strains, with *Cyanothece* sp. ATCC 51142 exhibiting the longest activity period, indicating differences in the carbon reserves of the strains and the possibility of the reserves being utilized for divergent pathways.

Unicellular diazotrophic cyanobacteria maintain a dynamic metabolic profile throughout the diurnal cycle. *Cyanothece* sp. ATCC 51142 was shown to be highly active during the dark phase of a day and night period, with the abundance of transcripts being highest at the early dark period. The rate of protein turnover was also thought to be higher in the dark period (Stöckel et al., 2008). Cellular activities during the dark comprise processes like respiration, nitrogen fixation, hydrogen production, and glycogen degradation. Amino acid biosynthetic pathways are also up-regulated during the dark period. During the light phase, processes like photosynthesis, carbon fixation, and glycogen synthesis are predominant. Highly regulated biochemical processes control the transition between the light and dark phases. Such traits make members of the genus *Cyanothece* of great interest where the influence of the circadian cycle and diurnal cycle on metabolism can be studied. This study reveals that unicellular diazotrophic cyanobacteria with the same genotypic background exhibit considerable diversity in the diurnal patterns of central metabolic processes. The variations appear to be controlled by intracellular metabolic signals specific to each strain, such as the intracellular concentration of carbon, nitrogen, or oxygen, which in turn control the ability of the cells to fix nitrogen. High rates of respiration, adequate supply of energy and reductants from efficient photosynthesis, and yet unknown components of the nitrogen-fixing machinery appear to be factors contributing to the unique aerobic hydrogen-producing ability of the genus *Cyanothece*.

**MATERIALS AND METHODS**

**Cell Growth**

*Cyanothece* sp. ATCC 51142 cells were grown in ASP2 medium (Reddy et al., 1993) in shaking flasks at 30°C under 30 μmol photons m⁻² s⁻¹ white light and ambient CO₂. All the other *Cyanothece* spp. strains were grown in BG11 medium (Allen, 1968) under similar growth conditions. For nitrogenase activity and hydrogen production studies, the *Cyanothece* spp. strains were grown in shaking flasks or bioreactors in ASP2 or BG11 medium (Reddy et al., 1993; Page et al., 2012), without supplemented NaN₃ at 30°C under 12-h light/12-h-dark cycles and 100 μmol photons m⁻² s⁻¹ white light. The growth curves under azotrophic and diazotrophic conditions were generated by sampling and measurement of the optical density of the cultures at 730 nm (O.D. 730) on a BioTek μQuant plate reader at an interval of 24 h for 7 d. The light micrographs and the absorption spectra of the strains were obtained from 6- to 7-d-old cultures grown in the presence of combined nitrogen sources. The images were obtained using the FluoView FV-1000 microscope (Olympus) and an Olympus DP71 color digital camera, and the spectra were obtained on an Olis DW-2000 spectrophotometer. The nitrogen-fixing *Cyanothece* spp. cultures used in this study were established by inoculating 0.25 volume of cultures (5-7 d old, O.D. 730 between 0.5 and 0.7) grown in ASP2 or BG11 medium (without combined nitrogen sources) under continuous light (30 μmol photons m⁻² s⁻¹ white light). This culture in turn was started by inoculating a one-tenth volume of cells (from 6- to 7-d-old cultures, O.D. 730 approximately 0.8) under 30 μmol photons m⁻² s⁻¹ continuous white light. For photomixotrophic growth analyses, cultures were supplemented with 50 μM glycerol.
Electron Microscopy

In preparation for electron microscopy, all cyanobacterial strains were grown in continuous 30 μmol photons m\(^{-2}\) s\(^{-1}\) while light at 30°C. *Cyanothece* sp. PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802 were harvested after 5 d directly from plates containing solid BG11 medium. Cells were collected from plates as cell paste and transferred directly to aluminum planchettes with 200-μm-deep wells. *Cyanothece* sp. ATCC 51142 was harvested as liquid culture grown in ASP2 medium after 6 d. Culture aliquots (25 mL) were centrifuged, and the cell pellet was resuspended in a small volume of medium and pipetted into planchettes with 200-μm-deep wells. All cells were fixed by ultra-rapid, high-pressure freezing using a Bal-Tec High Pressure Freezer (Bal-Tec). *Cyanothece* sp. PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802 samples were freeze substituted in 1% (v/v) glutaraldehyde plus 0.1% (w/v) tannic acid in acetone and embedded in Epon/Araldite. *Cyanothece* sp. ATCC 51142 samples were freeze substituted in 1% (v/v) glutaraldehyde plus 0.1% (w/v) tannic acid in acetone and embedded in Spurr and polymerized for 48 h at 60°C, followed by a slow thaw to room temperature. Thin sections were stained with uranyl acetate and lead citrate and then zero-loss imaged using a LEO 912 energy filter transmission electron microscope equipped with a ProScan digital camera.

Hydrogen Production, and Respiratory and Nitrogenase Activity Measurements

To determine the ratio of nitrogen fixation and hydrogen production, 20 mL of culture (from a 7-d-old, 12-h light/dark culture) was transferred at the beginning of the dark period from the bioreactors and/or shake flasks to air-tight glass vials (56 mL) and incubated in air under a light intensity of 100 μmol photons m\(^{-2}\) s\(^{-1}\) for 12 h. For anaerobic incubation, the glass vials were flushed with argon for 15 to 30 min. For experiments conducted over the diel cycle, 10-mL samples were collected every 2 h from bioreactors as well as shake flasks and incubated in 36-mL vials for 1 h in air under a light intensity of 100 μmol photons m\(^{-2}\) s\(^{-1}\). Nitrogenase activity of the cultures was determined using an acetylene reduction assay (Oda et al., 2005), following the protocol described by Bandypadhyay et al. (2010), and expressed in terms of the ethylene produced. Ethylene and hydrogen that accumulated in the head space of sealed culture vials were withdrawn with an air-tight syringe and quantified using an Agilent 6890N gas chromatograph (Bandyopadhyay et al., 2010). Respiratory activities of the cells were measured in photobioreactors equipped with an integrated Mettler-Toledo Clark-type oxygen electrode (Page et al., 2012). The vented bioreactors were operated in stirring mode to evaluate the changes in dissolved oxygen concentration of the cultures from photosynthetic and respiratory activities of the cells. The cultures were transferred to the bioreactors at the beginning of the log phase of growth and studied in batch culture mode under anaerobic conditions for 7 d. The optical density of the cultures did not change significantly over the 72-h period during which the cultures behaved similarly, as evident from the pattern of respiration and photosynthesis. After 3 d, the culture variations were observed in the cycling pattern and the cells showed signs of stress. Thus, data obtained over the 3-d period are presented here. Total chlorophyll \(a\) was extracted by methanol and quantified spectrophotometrically using an Olis DW2000 spectrophotometer. The chlorophyll content of *Cyanothece* sp. ATCC 51142 cultures grown without an external carbon source ranged between 0.5 and 2 μg mL\(^{-1}\), whereas cultures supplemented with glycerol had higher chlorophyll concentrations (2–5 μg mL\(^{-1}\)).

Nucleotide-Level Comparison of the *nif* Cluster in Unicellular Nitrogen-Fixing Strains

Conserved regions are identified using National Center for Biotechnology Information nucleotide BLAST version 2.2.22 (Altschul et al., 1997) with parameters dust:no, word_size:11, gapopen:5, gapextend:2, penalty:-3, and reward:2. In Figure 7, the conserved regions in each strain are aligned with the reference sequence from *Cyanothece* sp. ATCC 51142. Regions are colored based on the percentage of identical bases within each region. In addition, we identified homologs to 35 protein-coding genes present in this region using National Center for Biotechnology Information protein BLAST. In this analysis, two genes are called homologs if reciprocal BLAST resulted in (1) an E value of less than 1E-4, (2) a ratio between the length of the BLAST hit region and the length of the complete protein of greater than 2:3, and (3) a ratio between the raw score for two-protein BLAST and the raw score for “self-self” BLAST of greater than 1:3:5.

The NiFH phylogenetic tree was generated using the amino acid sequence of NiFH in *Cyanothece* sp. ATCC 51142 as the reference. Homolog gene sequences in 103 cyanobacterial strains were identified from the Integrated Microbial Genomes database of the Department of Energy-Joint Genome Institute (Markowitz et al., 2010). The ClustalX2 version 2.1 (Larkin et al., 2007) software package was used to perform complete alignment of the individual sequences, and the phylogenetic tree was generated using a neighbor-joining algorithm with default parameters. The resultant tree was rendered using the Archaeopteryx version 0.95bt software program (Han and Zmasek, 2009).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree for cyanobacteria strains based on the NiFH sequence.

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