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The Uptake Hydrogenase in the Unicellular Diazotrophic Cyanobacterium *Cyanothece* sp. Strain PCC 7822 Protects Nitrogenase from Oxygen Toxicity

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***Cyanothece* sp. strain PCC 7822 is a unicellular, diazotrophic cyanobacterium that can produce large quantities of H_2 when grown diazotrophically. This strain is also capable of genetic manipulations and can represent a good model for improving H_2 production from cyanobacteria. To this end, a knockout mutation was made in the *hupL* gene (Δ*hupL*), and we determined how this would affect the amount of H_2 produced. The Δ*hupL* mutant demonstrated virtually no nitrogenase activity or H_2 production when grown under N_2-fixing conditions. To ensure that this mutation only affected the *hupL* gene, a complementation strain was constructed readily with wild-type properties; this indicated that the original insertion was only in *hupL*. The mutant had no uptake hydrogenase activity but had increased bidirectional hydrogenase (Hox) activity. Western blotting and immunocytochemistry under the electron microscope indicated that the mutant had neither HupL nor NifHDK, although the *nif* genes were transcribed. Interestingly, biochemical analysis demonstrated that both HupL and NifH could be membrane associated. The results indicated that the *nif* genes were transcribed but that NifHDK was either not translated or was translated but rapidly degraded. We hypothesized that the Nif proteins were made but were unusually susceptible to O_2 damage. Thus, we grew the mutant cells under anaerobic conditions and found that they grew well under N_2-fixing conditions. We conclude that in unicellular diazotrophs, like *Cyanothece* sp. strain PCC 7822, the HupLS complex helps remove oxygen from the nitrogenase, and that this is a more important function than merely oxidizing the H_2 produced by the nitrogenase.**

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis, and they play a central ecological role due to their abundance and dispersal in a wide variety of habitats. They also have a highly versatile metabolism and can grow photoautotrophically, mixotrophically, and heterotrophically. Like other bacteria, some cyanobacteria can fix atmospheric N_2 to generate biologically available nitrogen in the form of ammonia. This process is catalyzed by the enzyme nitrogenase that also produces H_2 as a by-product of N_2 fixation (1–4). In addition to the nitrogenase, most cyanobacteria contain two other enzymes that can affect hydrogen levels, a bidirectional hydrogenase (encoded by the *hox* genes) and an uptake hydrogenase (encoded by *hupLS*). The bidirectional hydrogenase is a member of the NAD(P)-reducing class, which consists of the diaphorase subunit (encoded by *hoxEFU*) and hydrogenase subunits (*hoxHY*). The bidirectional hydrogenase is involved in fermentative hydrogen production, and one primary role is to use protons as final electron acceptors during cellular fermentation. The behavior of this enzyme is dependent on redox conditions: if low-potential electrons are plentiful, Hox is used to pass them on to protons to form H_2. Under oxidizing conditions, H_2 is split into protons and electrons can again be utilized for metabolism (5). The uptake hydrogenase is composed of small and large subunits, encoded by *hupS* and *hupL*, respectively. HupS mediates electron transport from the active site of the large subunit to redox partners and downstream reactions through a set of FeS clusters. HupL harbors the active site that contains the metals Ni and Fe. The uptake hydrogenase may reabsorb the H_2 produced by the nitrogenase (1), but it may have three other beneficial functions for the organism: (i) provide the organism with ATP via the oxyhydrogen (knallgas) reaction; (ii) remove oxygen from nitrogenase, thereby protecting it from inactivation; and (iii) supply reducing equivalents (electrons) to nitrogenase and other enzymes (1, 4, 6, 7).

Since both the uptake hydrogenase and nitrogenase enzymes are poisoned by oxygen, the cyanobacteria have developed a number of different strategies to protect these enzymes from inactivation. Certain N_2-fixing filamentous cyanobacteria can differentiate some cells into heterocysts, which synthesize a thick envelope that interferes with O_2 diffusion and in which photosystem II (PS II) is inactive (8, 9).Thus, these organisms, such as *Anabaena* sp. strain PCC 7120, produce a molecular environment in the heterocysts that is very low in oxygen and permits the functioning of nitrogenase.

Unicellular cyanobacteria, such as *Cyanothece* sp. strain ATCC 51142 and *Cyanothece* sp. strain PCC 7822, temporally separate N_2 fixation from photosynthesis. They perform photosynthesis during the day, during which time they evolve O_2 and fix CO_2. The fixed carbon is then stored in glycogen granules that are degraded during the night, and the carbohydrate is used as a substrate for respiration. Nitrogenase and the respiratory enzymes are produced during the night, and respiration reduces intracellular oxygen to water, in addition to providing energy for N_2 fixation. Thus, the two incompatible processes, O_2 evolution and N_2 fixation, are temporally separated, and large amounts of N_2 are fixed and H_2 produced in the dark. Once the stored carbohydrate is

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Cyanothece strains		
PCC 7822	Wild type	15
Δ*hupL* mutant	*Cyanothece* sp. strain PCC 7822 *hupL*::Km^r	This work
C-Δ*hupL* strain	Complementation strain of Δ*hupL*	This work
Plasmids		
Topo-TA	Cloning vector, Ap^r, *lacZ′*, *mcs*	Invitrogen
pRL448	Source of Nm/Km cassette, Ap^r, Km^r	16
phL	*hupL* PCR fragment cloned into Topo-TA	This study
phL-K	Nm/Km cassette inserted into EcoRI site of *hupL*	This study

TABLE 2 Primers used for PCR, semiquantitative PCR, and mutant construction

Primer	Sequence	Annealing temp (°C)
hupL7822MF(F1)	AGTGACACAAAGACCCACGAC	
hupL7822MR(R1)	AAGATGGCTATGTGGTCAACG	52
rnpBF	CGTGAGGATAGTGCCACAGA	
rnpBF	AAACGGGACCGGTAAAAGAC	55
hoxHF	GCTGAAGCCGGAATTAACAA	
hoxHR	ATTTGTAGCGGCATTTGTCC	53
hupLF2	AACGGTAAACCGATCAAACG	
hupLR2	CGGATGGGTCTTGATATTGG	53
nifHF	CAACACCAAAGCGCAAGTAA	
nifHR	GGATAGGCATAGCGAAACCA	54
nifDF	ATTTCCAAGAACGCGACATC	
nifDR	TCACGAACAGCATCGTTAGC	53
psbAF	CCCACCCTTCTGACAGCTAC	
psbAR	CTAACTGGTAAGGGCCACCA	52
NmKmF(F2)	TGCTCCTGCCGAGAAAGTAT	
NmKmR(R2)	ATTATCACGGGTAGCCAACG	53

consumed, the intracellular oxygen level goes up and the nitrogenase is proteolytically degraded (10).

Hydrogen is believed to be an important energy carrier for the future. A large number of organisms are able to produce H_2; however, only cyanobacteria and green algae produce this energy carrier from sun and water by the photosynthetic apparatus. During the last decade, there has been an increasing focus on these microorganisms as producers of biofuels. *Cyanothece* sp. strain PCC 51142 was shown to be a superior H_2 cyanobacteria producer when grown under aerobic conditions and then incubated under argon. The H_2 production rate reached 373 μmol/mg chlorophyll *a* (Chl a)/h under such conditions and over 400 μmol/mg Chl a/h when grown in the presence of 50 mM glycerol (11). On the other hand, the filamentous cyanobacterium *Anabaena variabilis* yields 39.4 μmol/mg Chl a/h. Similarly, an uptake hydrogenase-deficient mutant strain of *Anabaena variabilis* PK84 produced 1,670.6 μmol/mg Chl a/h when grown anaerobically with 25% N_2, 2% CO_2, and 73% Ar (12, 13). In filamentous cyanobacteria, the uptake hydrogenase has become a key candidate for inactivation in order to improve H_2 production. The H_2 production rate of the Δ*hupL* strain of *Nostoc* sp. strain PCC 7422 increased some 3-fold compared to the wild type to 80 μmol/mg Chla/h (14). However, the H_2 production activity of a Δ*hupL* mutant of *Nostoc punctiforme* 29133 produced 9 μmol/mg Chl a/h, in contrast to the trace amount in the wild type (15). Thus, the uptake hydrogenase may perform differently in different strains of cyanobacteria.

Our objective was to analyze the physiological and genetic processes vital for cyanobacteria to become effective producers of biohydrogen by the use of solar energy and water. The initial step was to construct and characterize an insertion mutant in the *hupL* gene to see how this would affect net H_2 production. The results in this study provide new insights into processes that directly or indirectly are of great importance for hydrogen metabolism, as well as for the relationship of the uptake hydrogenase to the nitrogenase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Stock cultures of *Cyanothece* sp. strain PCC 7822 (16) were grown under continuous light conditions (30 to 50 μmol photons m^{-2} s^{-1} at 30°C) in BG11 medium aerated by bubbling with air or on a shaker at 125 rpm. The medium for growth of the mutant Δ*hupL* strain was supplemented with 15 μg/ml kanamycin. For nitrate step-down experiments, *Cyanothece* sp. strain PCC 7822 cultures were inoculated in BG11 for several days and harvested, washed 3 times with nitrogen-free BG11 (BG11 NF) medium, and transferred to BG11 NF medium for 3 days. For anaerobic growth conditions, cultures were incubated in a 3.5-liter anaerobic jar with an Oxoid AnaeroGen atmosphere-generating system (Thermo Scientific, Cambridge, England).

Mutant construction. The PCR-amplified *hupL* gene (primer pair hupLMF and hupLMR) (Table 1) was cloned into a Topo-TA vector (Invitrogen Topo-TA cloning kit), and a neomycin/kanamycin cassette (Nm/Km) from pRL448 (17) was inserted into *hupL* at an EcoRI site (phL-K) and then sequenced. Single-strand PCR products from asymmetrical PCR of *hupL*::Nm/Km were transformed into *Cyanothece* cells (18), and single colonies were selected from BG11 plates supplemented with 15 μg/ml kanamycin. Asymmetric PCR with products carrying the *hupL* gene with a Km/Nm cassette insertion was conducted with primer hupLMF at 0.2 μM final concentration and hupLMR at 1:100 of this concentration to produce single-stranded DNA (ssDNA). The PCR product of ssDNA was boiled for 5 min and put on ice immediately for electroporation. The complementation strain of the Δ*hupL* mutant (C-Δ*hupL* strain) was obtained using the same method with selection on BG11 NF plates.

Confirmation of the mutant. PCR was performed using one primer located at *hupL* and the other primer at the Nm/Km cassette, with the genomic DNA (gDNA) of the Δ*hupL* mutant as the template. DNA sequencing was conducted on the PCR product from one colony, and the results indicated that the Nm/Km cassette was inserted at the EcoRI site of *hupL*.

Semiquantitative PCR. Total RNA was isolated after nitrate step down for 24 h. DNase I (Invitrogen DNase I, amplification grade) treatment and reverse transcription (SuperScript III RT; Invitrogen) was performed according to the manufacturer's instructions.

PCR was carried out to amplify regions of the genes for *rnpB* (22 cycles), *hoxH* (32 cycles), *hupL* (28 cycles), *nifH* (28 cycles), *nifD* (28 cycles), and *psbA* (30 cycles). The same conditions and number of cycles were used for genes from both the wild-type and Δ*hupL* strains. The *rnpB* gene was included, as this transcript is frequently used as a constitutively expressed control for gene expression. Primers and annealing temperatures are listed in Table 2.

Assays for nitrogenase, H_2 production, uptake hydrogenase, and bidirectional hydrogenase. (i) Nitrogenase activity. Nitrogenase activity was measured, as described previously (19), using an acetylene reduction assay and expressed in terms of the ethylene produced. Twelve ml of culture was incubated in 66-ml sealed glass vials at 50 μE/m^2/s light intensity at 30°C for 24 h under an atmosphere of 3 ml of acetylene with argon flushing. Gas samples of 200 μl were measured using a Hewlett Packard 5890 series II gas chromatograph according to the manufacturer's instructions.

(ii) H_2 production. H_2 evolution was measured with 45 ml of cultures in 66-ml bottles, sparged with argon, and incubated for 24 h under continuous light (light intensity, 50 μmol photons m^{-2} s^{-1}) at 30°C for 24 h. Gas samples of 200 μl were measured using a Hewlett Packard 5890 series II gas chromatograph (11, 18).

(iii) Bidirectional hydrogenase. After nitrate step down, wild-type and mutant cells were induced under microaerobic conditions in modified BG11 NF medium for 36 h (20). Cells were harvested and suspended in 50 mM Tris-HCl (pH 7.5) and broken twice in a French pressure cell, and total cell extraction was used in the assay. The proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM) was introduced, and Hox activity was measured for 1 h with reduced methyl viologen (MV) as described previously (20).

(iv) Uptake hydrogenase. After nitrate step down, cells were incubated in BG11 NF medium for 24 h before harvesting. The assay was performed essentially as described previously (20), with minor modifications. The cultures were centrifuged and washed in Tris-HCl (pH 7.5) buffer. After cells were broken two times in the French pressure cell, 2.7-ml samples were used in the assay. H_2 uptake was performed in the presence of phenazine methosulfate (PMS), and the reactions were allowed to proceed for 3 h in the dark. The reaction mixtures, sparged with argon, consisted of 50 mM Tris-HCl (pH 7.5), 3 mM PMS, and the cell supernatant. The H_2 uptake reaction was initiated by the addition of H_2 to a final concentration of 2.0% (vol/vol).

(v) Light microscopy. Cells were observed with a VWR Vista vision microscope, and light micrographs were obtained with a DV-1B digital camera.

(vi) Miscellaneous procedures. Total chlorophyll *a* (Chl a) was extracted by methanol and quantified spectrophotometrically in a PerkinElmer lambda 40 spectrophotometer. The concentration was determined by using the equation $[\text{Chl}] = 14.97(A_{678} - A_{750}) - 0.615(A_{620} - A_{750})$ by measuring light absorbance at 750, 678, and 620 nm (18). Protein concentrations were determined using the Bio-Rad protein assay according to the manufacturer's instructions.

Protein extraction, SDS-PAGE, and Western blotting. SDS-PAGE and Western blotting were performed as described previously (21). The anti-HupL antibody was diluted 1:1,500, and the NifH and NifD antibodies were diluted 1:1,000. Cells were broken in a French press, and the membrane and soluble fractions were prepared as described previously (22).

TEM sample preparation/immunogold labeling. *Cyanothece* sp. strain PCC 7822 and Δ*hupL* cells were grown in BG11 for 1 week, transferred to BG11 NF in continuous light, and recultured into fresh medium 24 h before harvesting. The cells were then grown under a 12-h light/12-h dark cycle for 3 days, and cultures were harvested when the levels of hydrogen uptake activity in the wild type exhibited a maximum. Cultures were then centrifuged to concentrate cells for high-pressure freezing in a Wohlwend HPF 01 high-pressure freezer (Technotrade, Manchester, NH). Planchettes were dipped in hexadecene and blotted prior to use. The cells were deposited on a glass slide, and small pieces were cut off and placed in the 0.2-mm planchettes (type A). They were covered with the flat side of a type B planchette. The loaded planchette sandwich was then frozen and split, and the side containing the sample was transferred to a cryovial containing the substitution fluid that had been precooled to liquid nitrogen temperature. Only one sample was placed in each cryovial, which then were placed in a Leica FS-U freeze substitution (FS) unit set to −90°C and permitted to substitute (FS) for ~48 h. During this time, the substitution fluid thawed to a liquid state and the planchette gradually sank into the fluid. Six planchettes were frozen for each sample. The substitution fluid consisted of acetone, 0.25% glutaraldehyde, and 0.2% uranyl acetate. After FS, the samples received a programmed warmup to −50°C during a 20-h period. The samples were then removed from the planchettes and infiltrated with a graded series of acetone and HM-20 resin, starting with 10% resin over 48 h, placed in embedding vials, and polymerized using UV light for 24 h at −50°C. Thin sections were cut using a Leica UC6 ultramicrotome (Mager Scientific, Inc., Dexter, MI). The samples for Fig. 5 were high pressure frozen, substituted in 2% OsO_4 plus 0.5% uranyl acetate in acetone, and embedded in Epon after warm-up.

The sections were blocked using blocking agent (1% BSA-c, 5% goat serum, 0.1% cold water fish gelatin in bovine serum albumin [BSA] buffer; Aurion BSA products from Electron Microscopy Sciences, Hatfield, PA) for 20 min at 25°C prior to incubating overnight at 4°C in the primary antiserum (HupL antiserum was diluted 1:150, and NifH antiserum was diluted 1:1,000) in 20 mM Tris, 150 mM NaCl, 0.5% bovine serum albumin (TBS-B). Grids were washed with TBS-T (TBS plus 0.3% Tween 20), incubated with the secondary antiserum diluted 1:1,000 in TBS-TB (TBS-B plus 0.3% Tween 20) for 1 h at room temperature, and washed in TBS-T and distilled H_2O. Grids were stained using 2% uranyl acetate in 70% ethanol for 10 min prior to imaging using a Philips CM-100 at 80 kV. Images were captured on Kodak S-063 film that was later scanned at 600 dpi to produce digital images. The difference in labeling with the antibody between the wild type and the mutant was statistically analyzed using the Student *t* test.

The primary antisera used for both Western blotting and transmission electron microscopy (TEM) were polyclonal antibodies raised against the large subunit of the uptake hydrogenase (HupL) of *Moorea producens* (formerly *Lyngbya majuscule*) (23) (provided by Sara Pereira, Porto, Portugal) and against NifH and NifD from *Azotobacter vinelandii* (provided by Dennis Dean, Virginia Polytechnic Institute and State University, Blacksburg, VA). Secondary antisera used were goat-anti-rat immunoglobulin G conjugated to 10-nm colloidal gold particles for HupL and goat-anti-rabbit IgG conjugated to 10-nm colloidal gold (BB International, Cardiff, United Kingdom; distributed by Ted Pella Inc., Redding, CA).

RESULTS

Construction and preliminary characterization of the *hupL* mutant. The single-stranded DNA technique and electroporation (18) were used to transform *Cyanothece* sp. strain PCC 7822 with kanamycin as the selective agent (Fig. 1A). We felt that this strategy would be specific for *hupL*, since the closest downstream open reading frame (ORF; encoding a maturation protease) had a 223-bp intergenic region after *hupL*, and it was unlikely that this insertion would cause polar effects on transcription. The Δ*hupL* mutant single colonies were picked and transferred to liquid culture with kanamycin. The mutant was verified by PCR with the gDNA of the Δ*hupL* strain (Fig. 1B). Primers pairs F1 and R2 are located in *hupL*, and F2 and R1 are in the Nm/Km cassette. The product of F2R1 is about 300 bp, that of F1R1 is 1.5 kb, and that of F2R2 is 1.3 kb. The morphology of the mutant cells as seen by phase-contrast microscopy was different from that of the wild-type cells. Mutant cells were more spherical and often had a volume over 1.5-fold that of wild-type cells (Fig. 1C). Most importantly, the Δ*hupL* mutant could not grow in BG11 NF medium either in liquid medium or on plates under aerobic conditions (Fig. 2, right). Because this was an unexpected phenotype, we decided to perform additional genetic experiments. This included making and studying additional Δ*hupL* mutants utilizing the same procedure at the same site, and all resulted in the same phenotype (data not shown).

Complementation of *hupL* and restoration of wild-type characteristics. To confirm that the *hupL* gene was the only gene modified in the Δ*hupL* mutant, the Δ*hupL* mutant was complemented by electroporation with single-stranded DNA bearing the wild-type *hupL* gene. Cells were selected on BG11 NF plates; *hupL* mutant cells gradually died, whereas only the complemented strain, which can fix nitrogen, survived. A complementation strain (C-5) was selected on BG11 NF plates and confirmed by

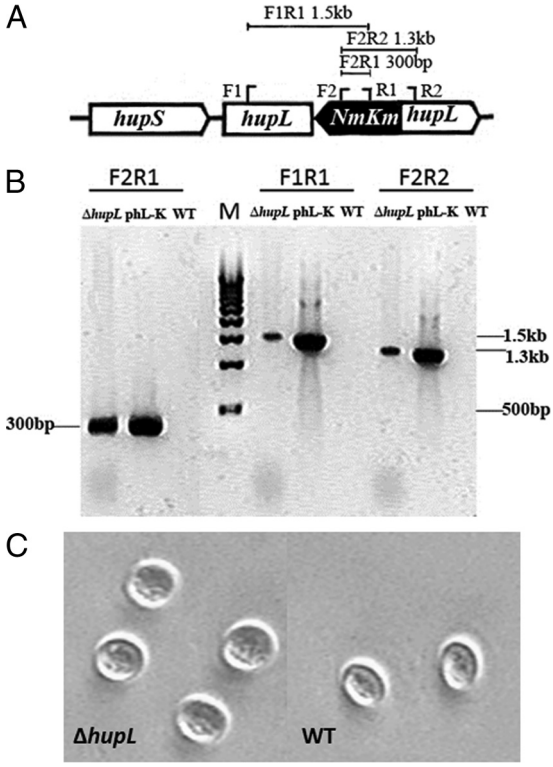

FIG 1 (A) PCR confirmation of the Δ*hupL* mutant showing the *hupLS* genomic region and the mutant construct. (B) PCR results with primer pairs F2R1, F1R1, and F2R2. Δ*hupL*, the gDNA of the Δ*hupL* mutant as the template for PCR. WT, the gDNA of the wild type as a negative control with the phL-K plasmid of the mutant construction as the template. M, 1-kb ladder. (C) Light micrographs of Δ*hupL* (left) and wild-type (WT; right) strains at ×400 magnification.

PCR on the transformed strain (data not shown). When the complemented cells were grown on BG11 NF plates, colonies grew nicely, with pigmentation and size that was comparable to that of the wild type (Fig. 2, right). The complementation experiment resulted in 40 colonies per plate at a frequency of 1×10^{-7} revertants/cell. Ten colonies from one plate were tested, and all 10 had wild-type characteristics. This ready construction of a complemented strain indicated that the loss of nitrogen-fixing ability and

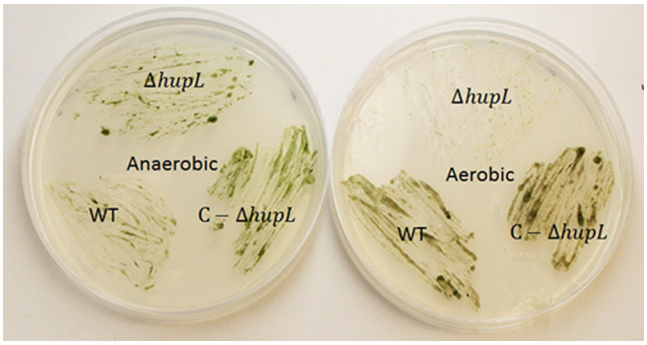

FIG 2 Growth conditions of the three strains on N_2-fixing plates under anaerobic (left) and aerobic (right) conditions. WT, *Cyanothece* sp. strain PCC 7822 wild type; Δ*hupL*, *hupL* deletion mutant strain; C-Δ*hupL*, complemented strain of the Δ*hupL* mutant.

TABLE 3 Comparison of H_2 production and nitrogenase activity after nitrate step down for 24 h in wild-type *Cyanothece* sp. strain PCC 7822, a Δ*hupL* mutant, and a complemented Δ*hupL* strain

Strain	Extent of H_2 production (μmol H_2/mg Chl a/day)	Extent of nitrogenase activity (μmol N_2 reduced/mg Chl a^1/day^1)
Wild type	2,474.4 ± 496.8	2,140.8 ± 340.8
Δ*hupL* mutant	84.0 ± 21.6	7.2 ± 2.4
C-Δ*hupL* strain	2,224.8 ± 434.4	1,759.2 ± 192.0

other physical properties were due solely to a deficiency in the uptake hydrogenase.

To further confirm this result, the nitrogenase activity and H_2 production were measured in the Δ*hupL* mutant, the wild type, and the C-Δ*hupL* complemented strain. When the Δ*hupL* mutant was transferred to BG11 NF medium, cells gradually turned yellowish and died. In order to assay the nitrogenase activity and hydrogen production ability in the nitrate-depleted medium, cells were first cultivated in BG11 medium for a few days, washed with BG11 NF medium 3 times, and then transferred to BG11 NF medium. After 24 h, the wild-type and C-Δ*hupL* strains showed normally high nitrogenase activity and hydrogen production, whereas the mutant had lost virtually all nitrogenase activity (Table 3). In addition, the mutant had low H_2 production levels that were similar to that produced only the bidirectional hydrogenase, as shown previously (11). It is evident that the C-Δ*hupL* complemented strain was performing these functions in a fashion similar to the wild type, whereas the Δ*hupL* mutant could not fix nitrogen or evolve hydrogen. We concluded that the loss of the uptake hydrogenase (HupLS) resulted in the loss of both functions. Therefore, the properties of a Δ*hupL* mutant strain were studied in more detail.

Transcription and translation of *nif*, *hup*, and *hox* genes in wild-type and Δ*hupL* strains. To investigate the expression of key genes, RNA was prepared from the mutant and wild-type cultures 24 h after nitrogen depletion. Semiquantitative PCR was carried out with the primers listed in Table 2. A control gene, *rnpB*, was used to demonstrate similar transcript levels in both strains. We observed that the transcript level of *hoxH* was significantly upregulated in the mutant strain relative to the wild type (Fig. 3). The transcript levels for the nitrogenase genes *nifH* and *nifD* remained at wild-type levels in the mutant after 24 h in nitrogen-depleted medium, which indicated that nitrogenase gene transcription was unimpaired. There were no *hupL* transcripts in the mutant cells, as expected. The expression of *psbA*, used as a control representing photosynthesis gene transcription, was similar in both strains. We then investigated the enzymatic capabilities of both Hox and Hup in the wild type and Δ*hupL* mutant as shown in Table 4. In the mutant, Hox activity more than doubled compared to that observed in wild-type cells, whereas uptake hydrogenase activity was not detectable in the mutant (Table 4). It should be noted that Hup activity was quite low, even in the wild type.

Western blotting for HupL and NifH. To examine the uptake hydrogenase and nitrogenase proteins in the wild-type and mutant cells, soluble protein, membrane protein, and total protein were isolated from wild-type and Δ*hupL* cells and assayed by Western blotting using polyclonal antibodies prepared against HupL and NifH. As show in Fig. 4, the HupL protein was detected in both soluble and membrane protein fractions, but the size was

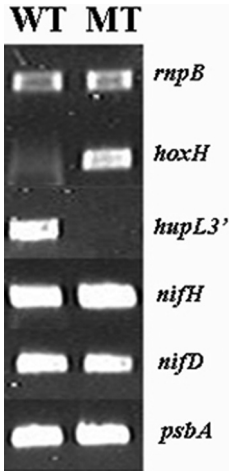

FIG 3 Semiquantitative PCR of related genes from *Cyanothece* sp. strain PCC 7822 wild-type (WT) cells and Δ*hupL* mutant (MT) cells that were grown under N_2-fixing conditions. RNA was isolated from cells that were grown first in nitrogen-replete media for 8 days and then resuspended in nitrogen-free media for 2 h in continuous light. hupL3′, primers located downstream of the antibiotic cassette Km/Nm.

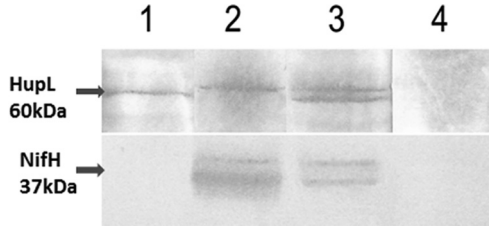

FIG 4 Western blot analysis of protein extracts of wild-type *Cyanothece* sp. strain PCC 7822 and the Δ*hupL* mutant. Cells were first grown in nitrogen-replete medium and then transferred into N_2-fixing conditions for 24 h under continuous light. The cells were then fractionated as described in Materials and Methods. Lane 1, soluble protein; lane 2, membrane protein; lane 3, total protein of the wild-type cells after step down for 24 h in nitrate-free medium; lane 4, total protein of Δ*hupL* cells after step down for 24 h in nitrate-free medium.

slightly different, as shown clearly for the wild type in lane 3. About half of the HupL protein was seen in the membrane fraction, and virtually all of the NifH protein appeared to fractionate with the membranes (mostly photosynthetic membranes). Finally, there was no HupL detectable in lane 4, representing the total protein of the Δ*hupL* mutant. Therefore, *hupL* was neither transcribed nor translated in nitrogen-free medium, whereas *nifH* was transcribed but either was not translated or was proteolytically degraded.

Ultrastructure and immunolocalization of HupL and NifH. Once we recognized that the *nifHDK* genes were transcribed but that neither NifH nor HupL proteins were present in cellular extracts, we wanted to confirm this *in situ*. Therefore, we prepared cells for analysis of ultrastructure and identification of the NifH and HupL proteins by immunocytochemistry via transmission electron microscopy. Cells were grown under nitrate-sufficient or nitrate-depleted conditions for 3 days in 12-h light-dark cycles, and cells were then prepared for TEM as described in Materials and Methods. Cells grown under nitrogen-depleted conditions for 3 days were harvested at approximately 1 h into the dark period. The high-pressure freezing procedure is the best current technique for preserving cellular integrity and gives a high level of antigenicity for specific proteins.

We first investigated cellular ultrastructure by including OsO_4 in the freeze substitution in order to visualize the photosynthetic membranes. Wild-type cells grown under nitrate-sufficient or nitrate-depleted conditions had plentiful photosynthetic membranes (Fig. 5A and B) and many small structures between the membranes that we have shown elsewhere are glycogen granules (24). The nitrate-replete cells also contained a few other granules, including cyanophycin granules (CG), that store nitrogen. Carboxysomes are often seen under both nitrate-replete and nitrate-deficient conditions, and some are seen prominently in Fig. 5B. The nitrate-depleted cells often showed the dark splotchy material in the center of the cell. This is the nucleoplasmic area with high concentrations of polyphosphate. One of the benefits of high-pressure freezing is that little water is extracted and the cells retain a more normal disposition of material. Therefore, the polyphosphate remains in the nucleoplasmic area rather than concentrating into a few large globules after chemical fixation. These granules often appear as holes in chemically fixed cells, since they are of a different density and often removed during microtomy.

Under nitrogen-sufficient conditions, the Δ*hupL* mutant grows like the wild type but clearly shows some ultrastructural differences (Fig. 5C). The photosynthetic membranes are stacked closer together and often cluster closer to the cytoplasmic membrane. These cells also begin accumulating granules, most of which are polyhydroxybutyrate (PHB) granules, while others are cyanophycin granules (Fig. 5C). These findings complement those from light microscopy that indicated that Δ*hupL* cells were somewhat larger, even under nitrogen-sufficient conditions. The mutant cells divide slightly slower and accumulate more C and N storage bodies. However, the major change was seen in the Δ*hupL* nitrate-depleted cells (Fig. 5D). These cells do not divide, although they do increase somewhat in biomass over the course of the first 2 days in this medium. However, it appears that most of the carbon is stored in the PHB granules that typically are located at the cell periphery, near the cytoplasmic membrane. In addition, photosynthetic membranes have decreased significantly.

We then analyzed cells that were prepared for immunocytochemistry; the same high-pressure freeze procedure was used but without osmium. Even though the procedure was optimized for immunocytochemistry and not intracellular ultrastructure, there are some important morphological differences that are immediately apparent. The wild-type cells shown in Fig. 6A and C once again showed photosynthetic membranes and a significant number of small, white granules between the membranes. These are the β-type glycogen granules that were seen in Fig. 5, and they are quite prominent in this procedure (24). The presence of a substantial number of glycogen granules is appropriate for cells that were harvested early in the dark period (D1) when nitrogenase is

TABLE 4 Methyl viologen-dependent bidirectional hydrogenase activity and uptake hydrogenase activity of wild-type *Cyanothece* sp. strain PCC 7822 and the Δ*hupL* mutant

	Activity (nmol H_2/mg Chl a/h) of:	
Strain	Hox	Hup
WT	29.7 ± 2.6	420 ± 110
Δ*hupL* mutant	74.9 ± 8.2	0

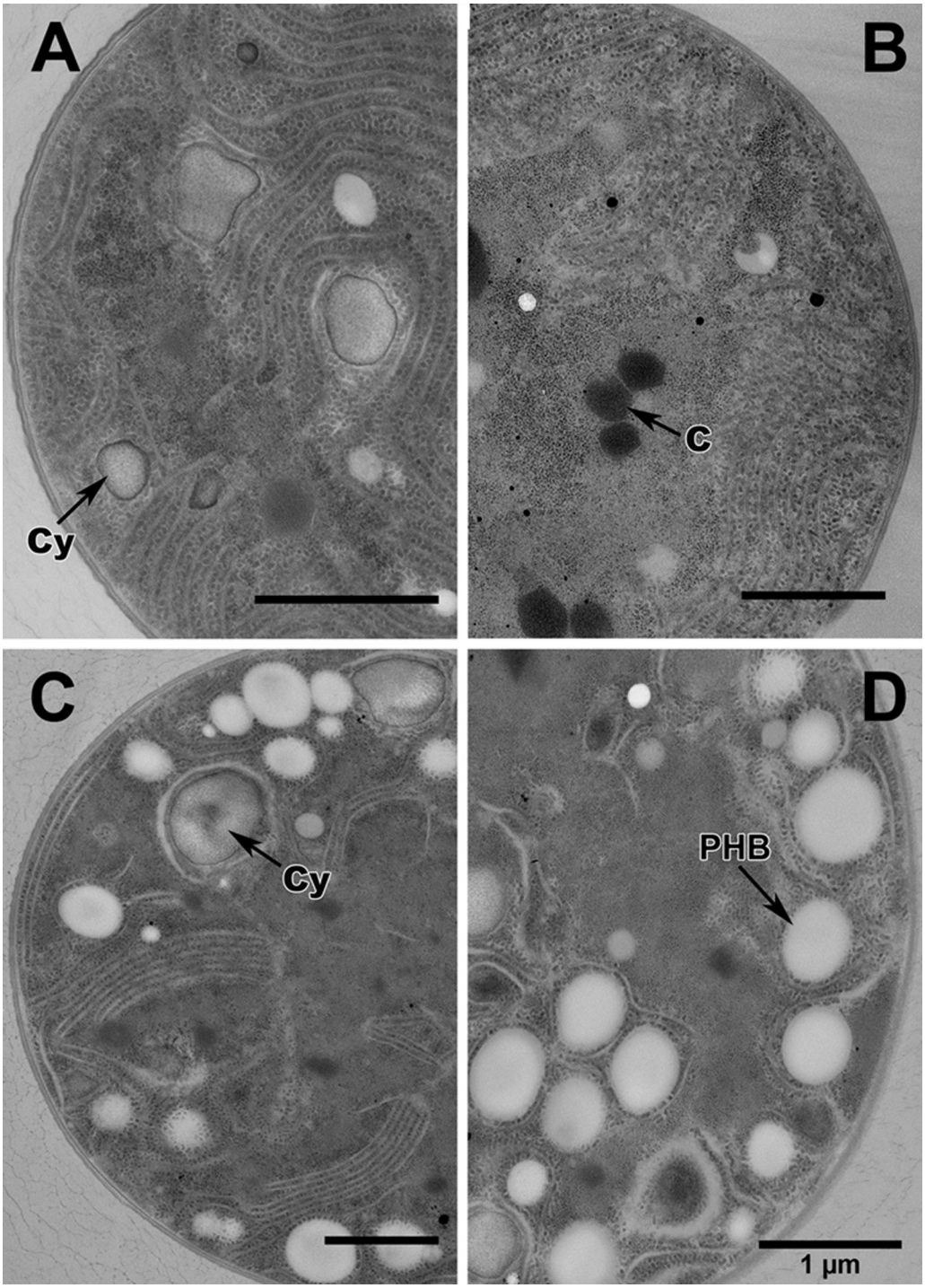

FIG 5 Transmission electron micrographs of high-pressure-frozen *Cyanothece* sp. strain PCC 7822 wild-type and Δ*hupL* strains grown under nitrate-replete conditions (A and C, respectively) and under nitrate-deficient conditions (B and D, respectively). PHB, polyhydroxybutyrate granules; Cy, cyanophycin granules; C, carboxysomes. The magnification bar represents 1 μm in each panel.

expressed and before the glycogen granules are degraded as a substrate for respiration. However, the Δ*hupL* cells had many different types of larger granules, as discussed for Fig. 5, which likely included CG and PHB granules (Fig. 6B and D). These are storage granules for nitrogen and carbon, respectively, and are an ultrastructural indication that the cells are not growing well. Without nitrogenase activity, the cells store carbon and some nitrogen rather than adding biomass and doubling. *Cyanothece* strains, unlike those of *Synechococcus* and *Synechocystis*, do not degrade phycobilisomes under nitrogen-deficient conditions and continue to store nitrogen (25). This process still occurred in the Δ*hupL* mutant.

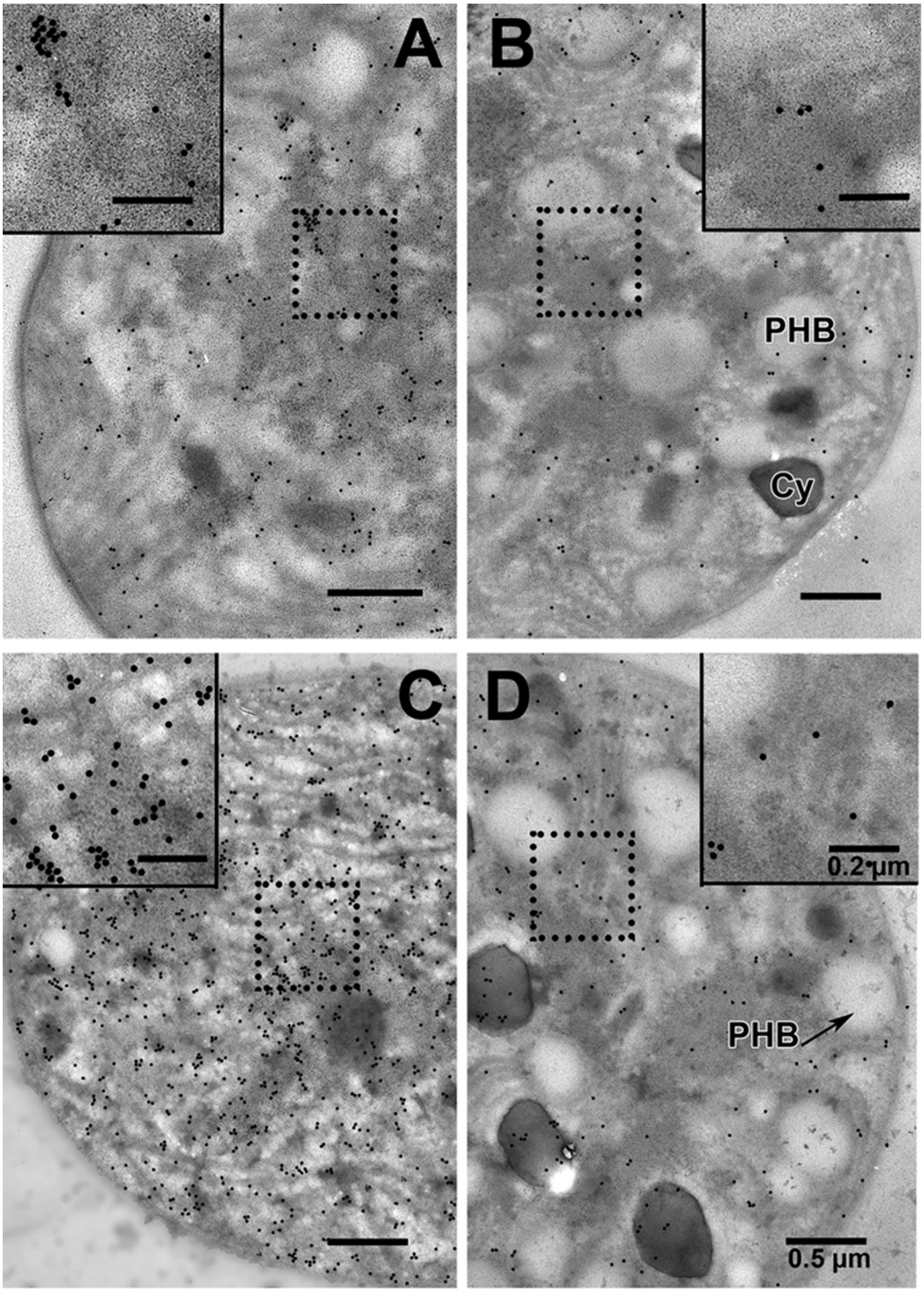

FIG 6 Immunocytochemistry of high-pressure-frozen *Cyanothece* sp. strain PCC 7822 wild-type and Δ*hupL* strains grown under nitrogen-deficient conditions. The material was prepared as described in Materials and Methods and then treated with antibody against HupL (A, wild type; B, Δ*hupL* mutant) and with antibody against NifH (C, wild type; D, Δ*hupL* mutant). The magnification bar represents 0.5 μm in each large panel. The dotted boxes represent the area of the cell that is expanded in the inset, and the magnification bar in each inset is 0.2 μm. PHB, polyhydroxybutyrate granules; Cy, cyanophycin granules.

The wild-type cells reacted moderately well with the HupL antiserum, and much of the label was located in the vicinity of the photosynthetic membranes and the glycogen granules. There was very little, if any, label on the Δ*hupL* cells. We consistently saw some gold particles in the Δ*hupL* cells, but the amount was variable. We quantitated the deposition of antibody on the wild-type and Δ*hupL* strains by counting gold label in 2-μm by 2-μm squares in two places on 10 separate cells. The wild type had 47.3 ± 9.4 gold particles per 4 μm^2, whereas the Δ*hupL* strain had 17.4 ± 4.6 gold particles per 4 μm^2. This difference was significant

at the $P = 0.05$ level by the Student *t* test. We conclude that there is little or no HupL in Δ*hupL* cells. We obtained similar results when we used the NifH antibody. As shown in Fig. 6C, we can identify hundreds of gold particles on the wild-type cells but only a few on the Δ*hupL* cells (Fig. 6D). Based on our enzymatic results, the NifH labeled with gold shown in Fig. 6D may not be active but is already targeted for proteolytic breakdown. It is evident that Δ*hupL* cells are also deficient in nitrogenase, likely because the loss of HupL leads to problems in protecting the nitrogenase complex from oxygen.

Anaerobic and aerobic incubation of wild-type, Δ*hupL*, and C-Δ*hupL* strains. Once it became evident that the Δ*hupL* mutant was unable to make or retain nitrogenase, we hypothesized that the absence of HupLS led to oxygen poisoning of the nitrogenase complex. Nitrogenase was then proteolytically degraded, as occurs late in the dark period after N_2 fixation (10, 19). Therefore, we established conditions for anaerobic growth to see if the Δ*hupL* strain could fix N_2 under such conditions. Cells were spread on BG11 NF plates and placed either under air or anaerobic conditions, which were established in a 3.5-liter anaerobic jar with an Oxoid AnaeroGen atmosphere-generating system. The Δ*hupL* mutant could not grow aerobically under N_2-fixing conditions but grew quite well under anaerobic conditions (Fig. 2, left). In addition, Δ*hupL* colonies under anaerobic conditions were greener and produced less EPS. Therefore, anaerobic conditions restored the ability of the Δ*hupL* strain to fix nitrogen and grow in the absence of combined nitrogen.

DISCUSSION

The nature of the phenotype of the Δ*hupL* mutant was clearly a surprise. Thus, we first ensured that the genotype was proper and that only a single gene was mutated. Importantly, the C-Δ*hupL* complementation strain demonstrated the recovery of both nitrogenase activity and H_2 production levels equivalent to those of the wild type, which suggested that the initial mutant was in a single gene (*hupL*). Once we were convinced that the physiological and morphological features of the mutant were due only to the loss of *hupL*, we could interpret all of the data in a holistic fashion and ask if oxygen sensitivity was at the heart of the problem. This was answered in the affirmative when the Δ*hupL* mutant grew well under anaerobic conditions in the light. Therefore, we concluded that the primary function of HupLS in *Cyanothece* sp. strain PCC 7822 is to protect the nitrogenase from oxygen toxicity, and the cells cannot fix N_2 aerobically when the uptake hydrogenase is absent. This result has important ramifications for the function of the hydrogenase and its relationship to the nitrogenase. Our protein results indicated that both the nitrogenase and uptake hydrogenase were dispersed throughout the cells, with a tendency to associate with intracellular membranes. Previous immunological studies indicated that nitrogenase in *Plectonema*, *Gloeothece*, and others are also uniformly distributed throughout all cells, showing no preferential association with a cell structure (21).

In general, the uptake hydrogenase is one of the major factors that influences hydrogen production. The main physiological function of the uptake hydrogenase is to reutilize and regain the H_2/electrons produced by the H_2 evolution through the nitrogenase. It reabsorbs H_2 produced by nitrogenase, especially in the presence of O_2 (1, 26). The uptake hydrogenase has been identified exclusively in strains that also have nitrogenase. In addition, the transcription of *hupLS* is induced only when the cells are fixing nitrogen (27), as we have shown in *Cyanothece* sp. strain 51142 (28) and in *Cyanothece* sp. strain PCC 7822 (D. Welkie, X. Zhang, and L.A. Sherman, unpublished data). Because of this relationship and the fact that the nitrogenase evolves at least one H_2 molecule per dinitrogen reduced to ammonia, it appeared logical to assume that HupLS functions to recycle this hydrogen to preserve the reducing power. However, some nitrogen-fixing strains do not possess an uptake hydrogenase (26), including *Cyanothece* sp. strain 7425 (16). Some of these strains do contain the bidirectional hydrogenase (Hox), but it has been shown that this enzyme does not oxidize hydrogen under N_2-fixing conditions (20). Our results with the mutant indicated that the *hox* genes are transcribed when HupLS is deficient, and that the Hox activity is greater in the mutant than in the wild type (Table 4). However, the activity is relatively low and very little protein has been found in our proteomics experiments (unpublished experiments). Thus, the Hox enzyme may oxidize some hydrogen, but this enzyme was obviously unable to rectify the loss of HupLS.

The Western blot demonstrated that both NifH and HupL have a membrane-associated component, and most of the membrane material in this fraction is photosynthetic thylakoids. NifH is mostly membrane bound, and HupL has a protein that migrates at a slightly higher molecular weight that associates with the membrane. In the usual classification of these enzymes, the first group is referred to as respiratory H_2-uptake enzymes, i.e., those localized to the bacterial cytoplasmic membrane, primarily involved with H_2 oxidation (29). The large surface area represented by the intracellular thylakoids can make this membrane an appropriate place for assembly of the nitrogenase complex, and it might require the hydrogenase for proper function. The possible functions of the uptake hydrogenase have been reviewed recently (5, 30), but there is much to be resolved. Biochemical experiments, especially those performed on hydrogenases from *Clostridium pasteurianum*, indicated that H_2 uptake was inhibited by CO, a known inhibitor of NiFe-hydrogenases (31). If O_2 is added, acetylene reduction by nitrogenase continued as normal, but the H_2 output was reduced. This suggested that at least a portion of the electrons from the HupLS reaction was passed into the respiratory chain and then to O_2. This inability to reduce O_2 in the absence of HupLS may be the proximal reason that nitrogenase cannot function in the *Cyanothece* sp. strain PCC 7822 Δ*hupL* mutant.

A number of previous *hupLS* mutants have been generated, mostly in heterocystous diazotrophs, such as the chemically induced *hup* mutant PK84 from *Anabaena variabilis* ATCC 29413 (13, 32). In this case, growth was performed under 25% N_2, 2% CO_2, and 75% Ar, an anaerobic atmosphere. Indeed, H_2 production was reduced significantly after the addition of 40 μM O_2 (32). Thus, although hydrogen production was improved significantly, this would not be useful for biohydrogen production. Our Δ*hupL* mutant could not grow under diazotrophic, aerobic conditions, but it could grow similarly to the wild type under diazotrophic, anaerobic conditions. Hup mutants in other cyanobacterial strains have generated a variety of results, and it appears that every strain has a different dependency on the functioning of the uptake hydrogenase. Another well-studied *hup* mutant is NHM5 from *Nostoc punctiforme* strain ATCC 29133 (15). Under aerobic conditions, this mutant grew somewhat less well than the wild type, had nearly identical nitrogenase activity, and produced H_2 much better than the wild type, which produced virtually no H_2. Thus, *N. punctiforme* reoxidizes the H_2 produced by nitrogenase, and the

loss of the HupLS led to net hydrogen production. This seems to be true for the filamentous, heterocystous cyanobacteria (see Ekman et al. [15] for details), and a different strategy might have developed in the unicellular diazotrophs. It should be noted that *Cyanothece* sp. strain 7425 that lacks the *hup* genes only fixes nitrogen under anaerobic conditions (33). Finally, the uptake hydrogenase was reported to play an important role in protecting nitrogenase from O_2, especially under carbon limitation conditions in *Frankia* sp. strain ArI3 (34).

We conclude that the uptake hydrogenase plays a particularly important role in the removal of O_2 from the nitrogenase in *Cyanothece* sp. strain PCC 7822, especially under our current cultivation conditions. By repeatedly growing the strain under more or less planktonic conditions, the strain produces less EPS and has lost some of its ability to fix N_2 under aerobic conditions. Thus, the removal of HupLS had an immediate effect on nitrogenase activity for aerobically grown cells. The biochemical results allow us to speculate that the hydrogenase is involved with the assembly of the nitrogenase oligomer at the photosynthetic membrane, and that excluding O_2 from nitrogenase at an early stage is important. Finally, the unicellular diazotrophic cyanobacteria and the heterocystous diazotrophs use the uptake hydrogenase in fundamentally different ways. This might relate to the necessary strategy of the unicellular organisms, because they have to contend with oxygen on a continuing basis without the advantage provided by the heterocyst.

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