Site-Directed Mutagenesis of the *Anabaena* sp. Strain PCC 7120 Nitrogenase Active Site To Increase Photobiological Hydrogen Production

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Cyanobacteria use sunlight and water to produce hydrogen gas (H₂), which is potentially useful as a clean and renewable biofuel. Photobiological H₂ arises primarily as an inevitable by-product of N₂ fixation by nitrogenase, an oxygen-labile enzyme typically containing an iron-molybdenum cofactor (FeMo-co) active site. In *Anabaena* sp. strain 7120, the enzyme is localized to the microaerobic environment of heterocysts, a highly differentiated subset of the filamentous cells. In an effort to increase H₂ production by this strain, six nitrogenase amino acid residues predicted to reside within 5 Å of the FeMo-co were mutated in an attempt to direct electron flow selectively toward proton reduction in the presence of N₂. Most of the 49 variants examined were deficient in N₂-fixing growth and exhibited decreases in their in vivo rates of acetylene reduction. Of greater interest, several variants examined under an N₂ atmosphere significantly increased their in vivo rates of H₂ production, approximating rates equivalent to those under an Ar atmosphere, and accumulated high levels of H₂ compared to the reference strains. These results demonstrate the feasibility of engineering cyanobacterial strains for enhanced photobiological production of H₂ in an aerobic, nitrogen-containing environment.

Photobiologically produced hydrogen gas (H₂) is a clean energy source with the potential to greatly supplement our use of fossil fuels (39). Whereas coal and oil are limited, cyanobacteria and eukaryotic microalgae can use inexhaustible sunlight as the energy source and water as the electron donor to produce H₂ (42). This gas is generated either by hydrogenases (52) or as an inevitable by-product of N₂ fixation by nitrogenases (49). In contrast to the reaction of hydrogenases which is reversible, nitrogenases catalyze the unidirectional production of H₂, although with substantial energy input in the form of ATP (47). Under optimal N₂-fixing conditions: N₂ + 8 e⁻ + 8 H⁺ + 16 ATP → H₂ + 2 NH₃ + 16 (ADP + Pᵢ), whereas, in the absence of N₂ (e.g., under Ar), all electrons are allocated to proton reduction: 2 e⁻ + 2 H⁺ + 4 ATP → H₂ + 4 (ADP + Pᵢ). Thus, one expects to be able to increase the H₂ production activity of nitrogenase by decreasing the electron allocation to N₂ fixation.

Nitrogenases are sensitive to inactivation by O₂; however, N₂-fixing cyanobacteria have developed mechanisms to protect these enzymes from photosynthetically generated oxygen (5). Of particular interest, *Anabaena* (also known as *Nostoc*) sp. strain PCC 7120 and some other filamentous cyanobacteria respond to combined-nitrogen deprivation by undergoing differentiation in which a subset of cells become heterocysts that provide a microaerobic environment, allowing nitrogenase to function in aerobic culture conditions. The nitrogenase-related (*nif*) genes are specifically expressed in heterocysts which lack O₂-evolving photosystem II activity and are surrounded by a thick cell envelope composed of glycolipids and polysaccharides that impede the entry of O₂ (56). Vegetative cells perform oxygenic photosynthesis and fix CO₂. Heterocysts obtain carbohydrates from those cells and, in turn, provide them with fixed nitrogen.

The molybdenum-containing nitrogenase of *Anabaena* sp. strain PCC 7120 consists of the Fe protein (encoded by *nifH*) and the MoFe protein (encoded by *nifD* and *nifK*). As in other organisms, the Fe protein is a homodimer containing a single [4Fe-4S] cluster and functions as an ATP-dependent electron donor to the MoFe protein. The latter is an α₂β₂ heterotetramer with each *nifD*-encoded α subunit coordinating the FeMo cofactor (FeMo-co; MoFe₇S₉X-homocitrate) that binds and reduces substrate, while α plus the *nifK*-encoded β subunits coordinate the [8Fe-7S] P-cluster (14). Additional *nif* genes are required for the biosynthesis of the metal clusters and maturation of the enzyme (40). The major *nif* gene cluster.

† Supplemental material for this article may be found at http://aem.asm.org/.

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of *Anabaena* sp. strain PCC 7120 undergoes two rearrangements in the heterocyst to yield nifB-fluxN-nifSUHDK-(1 ORF)-nifENX-(2 ORFs)-nifW-hexAB-fluxH (19).

One approach to increase H$_2$ production by nitrogenase is to enhance the electron flux to proton reduction and away from N$_2$ fixation. Although replacement of N$_2$ by Ar is effective for increasing H$_2$ production, this approach increases the operational cost for large-scale generation of H$_2$. Mutagenesis offers an alternative mechanism to overcome N$_2$ competition. The amino acid sequences of the MoFe ~subunit are highly conserved among different phyla (18). The V75I substitution in the suspected gas channel of NifD2 of *Anabaena variabilis* (equivalent to V70 in *A. vinelandii*) resulted in greatly diminished N$_2$ fixation, while allowing for H$_2$ production rates (under N$_2$) that were similar to those of wild-type cells under Ar (55). Significantly, however, the nonheterocyst nitrogenase of this strain, which is expressed mainly in vegetative cells under anaerobic conditions, is incompatible with O$_2$-evolving photosynthesis and thus requires continuous anaerobic conditions along with a supply of exogenous reducing sugars for H$_2$ production. Substitutions of selected amino acids in the vicinity of the FeMo-co active site within *Azotobacter vinelandii* nitrogenase were shown to eliminate or greatly diminish N$_2$ fixation while, in some cases, allowing for effective proton reduction (2, 10, 17, 27, 36, 44, 45, 48). Therefore, certain amino acid exchanges near FeMo-co might produce variant MoFe proteins in heterocyst-forming *Anabaena* that redirect the electron flux through the enzyme preferentially to proton reduction so as to synthesize more H$_2$ in the presence of N$_2$ in an aerobic environment.

To examine whether *Anabaena* sp. strain PCC 7120 nitrogenase can be modified to increase photobiological H$_2$ production by effecting such a redirection, we evaluated *in vivo* H$_2$ production and acetylene reduction rates of a series of cyanobacterial *nifD* site-directed mutants. We mutated six NifD residues (Fig. 1) predicted to lie within 5 Å of FeMo-co to create 49 variants using an *Anabaena* ∆NifΔHup (previously denoted ∆hupL) parental strain that lacks both an intact nifD and an uptake hydrogenase (34). In an atmosphere containing N$_2$ and O$_2$, several mutants exhibited significantly enhanced rates of *in vivo* H$_2$ production and accumulated high levels of H$_2$ compared to the reference strains.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The strains and plasmids used in the present study are described in Table S1 in the supplemental material. Cyanobacterial strains were grown in an 8-fold dilution of Allen and Arnon (AA/8) liquid medium or nondiluted AA agar (1) with (or without) added nitrate on a rotary shaker at 30°C. *Azocystis* strains were grown in an 8-fold dilution of Allen and Arnon (AA/8) liquid medium or nondiluted AA agar (1) with (or without) added nitrate on a rotary shaker. Medium or nondiluted AA agar (1) with (or without) added nitrate on a rotary shaker. *Azocystis* strains were grown in an 8-fold dilution of Allen and Arnon (AA/8) liquid medium or nondiluted AA agar (1) with (or without) added nitrate on a rotary shaker. Material strains were grown in lysogeny broth (LB) medium at 37°C. The media for the present study are described in Table S1 in the supplemental material. Cyanobacterial strains were grown in an 8-fold dilution of Allen and Arnon (AA/8) liquid medium or nondiluted AA agar (1) with (or without) added nitrate on a rotary shaker at 30°C. *Escherichia coli* strains were grown in lysogeny broth (LB) medium at 37°C. The media for the present study are described in Table S1 in the supplemental material. Cyanobacterial strains were grown in an 8-fold dilution of Allen and Arnon (AA/8) liquid medium or nondiluted AA agar (1) with (or without) added nitrate on a rotary shaker at 30°C.

**Cloning of the na region.** The na region was achieved by combining fragments, initially a 2.7-kb EcoRV-[nifU]-[nifH]-[nifD]-5'EcORI fragment (see pHM1) and a 5.9-kb EcoRV-[nifD3]-[nifK]-ORF-[nifEN]-[nifX]-3'EcORI fragment that is adjacent to it in heterocyst genomic DNA. In vegetative cells, the latter fragment normally bears an excision element that is excised from the chromosome in heterocysts (19). To obtain the 5.9-kb fragment, plasmid pHM6 was first integrated at the nifX/N locus in the *Anabaena* wild-type chromosome to create mutant AnNifNX. Genomic DNA was extracted from heterocysts isolated from this mutant, digested with EcoRI, recircularized by self-ligation, and transformed into *E. coli* XL1-Blue MRF* with selection on an LB agar plate containing 50 µg of kanamycin (Km) ml$^{-1}$. A transformant obtained a desired plasmid, denoted pHM7. The insert in pHM7, combined with pHM1, yielded pHM10 and was then reduced in size, producing pHM11. Because it was unclear whether the 556-bp promoter sequence upstream of nifH in pHM11 would suffice for the wild-type activity of nitrogenase (4), the region upstream of nifH was elongated, yielding pHM13.

The *Anabaena* strain into which mutated versions of nifD were transferred for testing was denoted ∆NifΔHup. Its provenance from the ∆Hup mutant by mutation with plasmid pHM27 is shown in Fig. S2. Single and double recombinants were screened as previously described (33, 34). The deletion of nifHDK-ORF in the ∆NifΔHup chromosome was confirmed by Southern blotting using probes for nifUH, nifD, and the npt (Km'/Nm') gene (34), as well as by PCR with the primer pairs nifD-F/nifD-R2, nifK-F/nifK-R, and nifHKD-ORF/nifHKD-ORF-R (data not shown).

To facilitate replacement of nifD with its mutated versions and for ease of sequencing to confirm the presence of the introduced mutations (and absence of unwanted mutations), a BamHI site was introduced into the nifD-nifK intergenic region. To this end, the sequence GGAACC was changed to GGATCC by overlap extension PCR (21): two PCR fragments, separately generated with the primer pairs nifD-F/nifD-R2, nifK-F/nifK-R, and nifHKD-ORF/nifHKD-ORF-R on template pHM13, were used as megaprimer to fuse these fragments. The product, amplified by PCR with the primer pairs nifD-F/nifD-R2, nifK-F/nifK-R, and nifHKD-ORF/nifHKD-ORF-R on template pHM13, was used as megaprimer to fuse these fragments. The product, amplified by PCR with the primer pairs nifD-F/nifD-R2, nifK-F/nifK-R, and nifHKD-ORF/nifHKD-ORF-R on template pHM13, was used as megaprimer to fuse these fragments.
FIG. 2. Construction of the ΔNifΔHup, complemented, and nifD site-directed mutant strains of Anabaena sp. strain PCC 7120. A region of the nifHDK-ORF genes was deleted from the parental ΔHup chromosome by gene replacement through a double-crossover event with pHM27 (top). The ΔHup strain used for this construction lacks an 11-kb nifD element containing the ntsA recombinase gene, unlike the wild-type strain with the nifD interruption (19). After verification of the deletion and complete segregation of the nifHDK-ORF region in the selected ΔNifΔHup mutant, plasmids containing the nifHDK-ORF, with or without a site-directed mutation in nifD, was integrated at the nifU3’-nifH5’ locus into the deletion mutant chromosome through a single-crossover event, yielding strains complemented by wild-type nifH, nifD, nifK, and ORF(all1439) genes or by variants whose nifD genes have site-directed mutations, respectively (middle). Homologous recombination occurred preferentially between the nifU3’-nifH5’ regions rather than between the ORF3’-nifE5’ regions due to the longer sequence length of the former (1,136 bp) than the latter (673 bp). The genome organization of the resulting single-crossover recombinants is shown at the bottom.
RESULTS

The residues targeted for mutagenesis in Anabaena sp. strain PCC 7120 nitrogenase (Fig. 1) were identified after mapping the cyanobacterial NifD and NifK sequences onto the high-resolution (1.16 Å) structure of *A. vinelandii* MoFe protein (PDB file 1M1N) (14). The corresponding sequences of the two microorganisms are 68 and 55% identical, with even greater identity in the region near the active site (47 of 48 residues identical within 8 Å of Fe-Mo-co). The structure predicts that portions of 19 residues, all highly conserved and encoded by nifD, lie within 5 Å of Fe-Mo-co. Residues C275 and H442 of *A. vinelandii* nitrogenase anchor Fe-Mo-co to the polypeptide by coordinating its terminal Fe and Mo atoms, respectively, and residues 355 to 360 (IGGFLRP) in the *A. vinelandii* enzyme have been suggested to be important for proper insertion of Fe-Mo-co into the protein (11). Therefore, the corresponding residues in *Anabaena* NifD (C282, H449, and residues 362 to 367; VGGGLRP) were left unchanged. In contrast, we carried out an extensive survey of changes to residues Q193, H197, R284, and F388 in the *Anabaena* enzyme, equivalent to residues (Q191, H195, R277, and F381) that were previously substituted in a limited fashion in the *A. vinelandii* protein (17, 27, 36, 44, 45, 48). Two additional residues lying within 5 Å of Fe-Mo-co in the *A. vinelandii* enzyme (Y229 and S278) were substituted in the *Anabaena* sequence (Y236 and S285). Each of the selected six residues was replaced by nonpolar, polar, or charged residues by using a parental *Anabaena* strain with ΔNif and ΔHup mutations (34), the latter to eliminate H₂ uptake. In all, 49 NifD variants were constructed: 8 for Q193, 13 for H197, 7 for Y236, 9 for R284, 8 for S285, and 4 for F388.

**Diazotrophic growth and H₂ production rates.** The 49 variants, the parental ΔNifΔHup and ΔHup strains, and the control AnNifΔHup strain (a wild type-nifHDK-ORF-complemented variant of the ΔNifΔHup mutant) were tested for diazotrophic growth at low light intensity (20 μmol photons m⁻² s⁻¹) on AA agar medium (1) lacking combined nitrogen. Strains shown to be capable of diazotrophic growth were analyzed for their rates of growth (see Fig. S1 in the supplemental material), and their specific growth rates were tabulated (Table 1). The control AnNifΔHup strain displayed essentially normal diazotrophic growth that was only slightly reduced from the parental ΔHup strain that was its predecessor, indicating that after transformation and single-crossover recombination the plasmid carrying the wild-type nifHDK-ORF genes was able to...

| Strain | N₂-fixing specific growth rates (h⁻¹) | Avg H₂ production rate (μmol mg of Chl a⁻¹ h⁻¹) SD | Avg percentage of rates under Ar relative to those under air ± SD |
|--------|--------------------------------------|-------------------------------------------------|---------------------------------------------------------------|
|        |                                      |                                                 |                                                               |
|        |                                      | (Under Ar)                                      | (Under air)                                                   |
| Q193G  | –                                    | 9.1 ± 1.3                                       | 2.4 ± 0.3                                                     |
| Q193A  | 0.21                                 | 20.8 ± 1.6                                      | 10.0 ± 0.4                                                    |
| Q193S  | –                                    | 20.3 ± 1.5                                      | 19.7 ± 3.8                                                    |
| Q193V  | –                                    | 5.0 ± 0.6                                       | 0.49 ± 0.08                                                   |
| Q193L  | –                                    | 20.9 ± 2.3                                      | 20.2 ± 1.8                                                    |
| Q193N  | –                                    | 16.0 ± 2.5                                      | 3.5 ± 0.5                                                     |
| Q193H  | –                                    | 16.9 ± 2.0                                      | 21.4 ± 0.3                                                    |
| Q193K  | –                                    | 9.1 ± 1.4                                       | 7.6 ± 1.9                                                     |
| H197G  | –                                    | 17.0 ± 3.2                                      | 11.4 ± 2.8                                                    |
| H197A  | –                                    | 2.8 ± 0.9                                       | 0.79 ± 0.3                                                    |
| H197A  | –                                    | 4.1 ± 0.4                                       | ND                                                            |
| H197T  | –                                    | 21.3 ± 1.9                                      | 20.7 ± 3.8                                                    |
| H197L  | –                                    | 14.9 ± 4.8                                      | 7.9 ± 2.9                                                     |
| H197F  | –                                    | 15.4 ± 3.8                                      | 14.5 ± 2.9                                                    |
| H197Y  | –                                    | 7.6 ± 3.3                                       | 4.7 ± 2.4                                                     |
| H197D  | –                                    | 10.0 ± 2.9                                      | 5.9 ± 1.9                                                     |
| H197E  | –                                    | 3.5 ± 0.8                                       | 1.8 ± 1.1                                                     |
| H197N  | –                                    | 10.6 ± 4.6                                      | 2.6 ± 1.6                                                     |
| H197Q  | –                                    | 2.0 ± 3.6                                       | 10.3 ± 3.1                                                    |
| H197K  | –                                    | 5.6 ± 1.8                                       | 4.1 ± 2.3                                                     |
| H197R  | –                                    | ND                                              | ND                                                            |
| Y236A  | –                                    | 10.9 ± 3.8                                      | 1.0 ± 0.3                                                     |
| Y236T  | 0.16                                 | 20.8 ± 0.9                                      | 12.3 ± 0.7                                                    |
| Y236M  | 0.062                               | 14.2 ± 3.0                                      | 6.6 ± 1.7                                                     |
| Y236F  | 0.22                                 | 15.7 ± 1.5                                      | 5.1 ± 0.3                                                     |
| Y236D  | 0.22                                 | 10.2 ± 3.9                                      | 2.7 ± 1.3                                                     |
| Y236N  | 0.15                                 | 15.1 ± 0.9                                      | 5.7 ± 0.7                                                     |
| Y236H  | 0.18                                 | 16.3 ± 4.9                                      | 7.3 ± 3.4                                                     |
| R284T  | –                                    | 9.4 ± 2.0                                       | 8.1 ± 2.1                                                     |
| R284C  | –                                    | 11.7 ± 2.2                                      | 7.9 ± 2.6                                                     |
| R284L  | –                                    | 13.8 ± 1.6                                      | 4.3 ± 1.4                                                     |
| R284F  | –                                    | 16.4 ± 1.6                                      | 3.1 ± 1.1                                                     |
| R284Y  | –                                    | 2.1 ± 0.8                                       | 0.48 ± 0.24                                                   |
| R284E  | –                                    | 1.9 ± 0.6                                       | 0.79 ± 0.32                                                   |
| R284O  | –                                    | 10.2 ± 2.7                                      | 5.8 ± 2.5                                                     |
| R284H  | –                                    | 16.0 ± 2.9                                      | 19 ± 3.3                                                      |
| R284K  | 0.087                                | 15.6 ± 0.7                                      | 7.9 ± 1.6                                                     |
| S285G  | –                                    | 8.4 ± 1.7                                       | 2.5 ± 1.2                                                     |
| S285A  | 0.16                                 | 16.1 ± 2.9                                      | 6.1 ± 1.4                                                     |
| S285T  | 0.17                                 | 19.9 ± 1.4                                      | 9.5 ± 0.8                                                     |
| S285C  | 0.17                                 | 22.3 ± 3.1                                      | 6.8 ± 1.6                                                     |
| S285M  | –                                    | ND                                              | ND                                                            |
| S285D  | –                                    | ND                                              | ND                                                            |
| S285N  | –                                    | 10.3 ± 1.4                                      | 14.0 ± 0.4                                                    |
| S285Q  | –                                    | ND                                              | ND                                                            |
| F388A  | –                                    | 11.8 ± 2.3                                      | 6.1 ± 1.8                                                     |
| F388T  | –                                    | 2.7 ± 0.6                                       | 1.4 ± 0.5                                                     |
| F388Y  | –                                    | 1.8 ± 0.4                                       | 0.4 ± 0.3                                                     |
| F388H  | –                                    | 12.6 ± 1.8                                      | 16.0 ± 3.6                                                    |

* Values are averages of three to six independent experiments, each performed with replicate or triplicate samples. H₂ production rates may be underestimated in some strains due to partial decomposition of Chl a (see Table 2). The strains indicated in boldface were studied in H₂ accumulation experiments.

**The ability to grow diazotrophically initially was tested by growth on AA agar plates lacking combined nitrogen for about 1 month. Cultures capable of diazotrophic growth were further analyzed to obtain growth curves in liquid culture as depicted in Fig. S1 in the supplemental material. The specific growth rates obtained from that experiment are tabulated here. A replicate experiment yielded analogous results. ND, not detected.**
restore the N₂ fixation phenotype to the ΔNifAHup deletion mutant. Only 9 NifD variants were capable of significant diazotrophic growth, with 1- to 3-day variations in the lag period before exponential growth was observed. The Q193A and Y236F variants were unchanged from the control AnNifAHup strain, only very small reductions in specific growth rates were observed for five variants (Y236T, Y236H, S285A, S285T, and S285C), and clearly decreased rates were observed for the Y236M and R284K mutants. The other NifD variants, including all H197 variants, were incapable of diazotrophic growth. In contrast, all of the strains grew normally in the presence of nitrate.

The in vivo H₂ production rates of the NifD variants and two reference strains were measured at 2 and 3 days after combined-nitrogen step-down under Ar or air (Table 1 and Fig. 3; see also Table S3 in the supplemental material). These tables also list the air/Ar H₂ production rate percentages as a rough estimate of the effects of the competing N₂ substrate on the variant nitrogenases. The ΔHup and AnNifAHup strains were similar in exhibiting rates under air that were 30 to 50% of those under Ar, a finding consistent with inhibition of H₂ production by N₂, as is typical of the wild-type nitrogenase (49). Some variants produced H₂ at rates similar to the reference strains for the two conditions, also indicating inhibition of H₂ production by N₂, while others possessed highly diminished or undetectable levels of proton reduction. Of greater interest, certain of the NifD variants exhibited H₂ production rates under air that were comparable to those under Ar, suggesting that N₂ does not greatly inhibit H₂ production by these mutant nitrogenases. The H₂ production rates for some variants under air were dramatically greater than the reference strains, in several cases reaching values similar to those of the reference strains under Ar when measured on a per-Chl a basis. As described in the section on quantification of CDW versus Chl a content, this approach may overestimate H₂ production rates in some cases where Chl a is partially degraded. Nevertheless, three variants (Q193H, R284H, and F388H) exhibited equivalent or higher rates under air (or 100% N₂; see Table S4 in the supplemental material) than under Ar.

Acetylene reduction rates. Table S5 in the supplemental material reports the rates of reduction of acetylene (12% in Ar) to ethylene and ethane by the 49 NifD variants and the two reference strains at 2 and 3 days after nitrogen step-down. The reduction of acetylene to ethylene often is used as a surrogate to assay nitrogenase activity, and the data indicate a wide range of such activity in the NifD variants. Notably, whereas cells...
containing the wild-type nitrogenase catalyzed only the two-electron reduction of acetylene, 18 of the 49 NifD mutants reduced this substrate to both ethylene and ethane, the latter requiring a four-electron reduction. For most of the ethane-producing variants, this gas was detected as only a minor product; however, the Q193K and H197N variants produced ethane at nearly one-half and one-quarter the rates, respectively, of ethylene formation.

Correlations among the abilities to fix N₂, produce H₂, and reduce acetylene to ethylene. To more readily compare the NifD variants in terms of their N₂ fixation phenotypes, H₂ production rates under Ar, and rates of acetylene reduction to ethylene, the data are combined in Fig. 3. The nine variants capable of growing well diazotrophically (Q193A, Y236T, Y236M, Y236F, Y236H, R284K, S285A, S285T, and S285C) all exhibited relatively high rates of acetylene reduction activity and large rates of H₂ production under Ar (62 to 114% and 82 to 128%, respectively, of their predecessor ΔHup strain). In contrast, 15 variants (H197G, H197T, H197L, H197Q, Y236A, Y236D, Y236N, R284T, R284C, R284L, R284F, R284Q, R284H, S285G, and F388A) were severely impaired in or incapable of diazotrophic growth, and yet they retained ~50% or more of the ΔHup strain rates of both acetylene reduction and H₂ production under Ar. Another 12 variants (Q193G, Q193S, Q193L, Q193N, Q193H, Q193D, H197E, H197F, H197Y, H197N, S285N, and F388H) accumulated nearly half or more of the ΔHup strain H₂ production rate under Ar, but their acetylene reduction rates were well below 50% of the ΔHup strain.

The remaining 13 variants reached less than 40% of the rates for either acetylene reduction or H₂ production compared to the ΔHup strain; only four strains (H197R, S285M, S285D, and S285Q) were essentially inactive for these activities. In general, changes in the FeMo-co environment more greatly affected acetylene reduction rates than H₂ production rates under Ar. Less acetylene reduction can account for an increase in H₂ under Ar. Another 12 variants (Q193G, Q193S, Q193L, Q193N, Q193H, Q193D, H197E, H197F, H197Y, H197N, S285N, and F388H) exhibited nearly half or more of the ΔHup strain H₂ production rate under Ar, but their acetylene reduction rates were well below 50% of the ΔHup strain.

The remaining 13 variants reached less than 40% of the rates for either acetylene reduction or H₂ production compared to the ΔHup strain; only four strains (H197R, S285M, S285D, and S285Q) were essentially inactive for these activities. In general, changes in the FeMo-co environment more greatly affected acetylene reduction rates than H₂ production rates under Ar. Less acetylene reduction can account for an increase in H₂ production, as reported previously using the A. vinelandii enzyme (17, 44). The striking exception to this generalization is the F388T strain, which exhibited less than 20% of the control H₂ production rate while retaining nearly 40% of acetylene reduction rate.

Accumulation of H₂ in atmospheres of Ar or N₂. Eleven NifD variants were selected for long-term H₂ accumulation studies. These mutants exhibited substantial increases over the two reference strains in their air/Ar H₂ production rates and most demonstrated increased rates under air compared to the ΔHup strains on the basis of Chl a (Table 1; see also Table S3 in the supplemental material). The strains were incubated in sealed vials using an atmosphere of Ar + 5% CO₂ or N₂ + 5% CO₂ and assayed for H₂ accumulation over 7 days (Fig. 4). All selected variant cultures produced H₂ continuously from 1 day to 4 to 6 days, at which point the H₂ concentrations leveled off at ca. 1 to 8%, depending on the cultures and conditions utilized. The H197Q and Y236T cultures exhibited profiles similar to those of the two reference strains under Ar + CO₂ and reached the highest maximum levels of H₂ (ca. 7 to 8%), which is consistent with the observation that these four strains had similar phenotypes with respect to H₂ production rates under Ar and air (Table 1; see also Table S3 in the supplemental material). However, when the reference strain cultures were grown under N₂ + 5% CO₂, the H₂ gas concentrations in the headspace leveled off at <1%, whereas all variant cultures accumulated significantly higher levels of H₂. Of the variants tested, the R284H culture exhibited the most dramatically increased levels of accumulated H₂ compared to the reference strain cultures when grown under N₂. This mutant strain reached 87% (two independent experiments) of the levels of H₂ accumulated by the reference strains under Ar at 5 to 7 days. The percentage of H₂ accumulated under N₂ versus that under Ar was compared for all 11 variant and 2 control strains at each time point in Table S6 in the supplemental material.

With the exception of the H197F, H197Q, Y236T, and reference strains, the selected variant cultures accumulated approximatively the same levels of H₂ under Ar as under N₂. These results are consistent with their similar H₂ production rates in...
that the mutant strains contained comparable levels of Chl

The hypothesis and to allow comparison with such measurements regardless of the different incubation conditions. To test this

H197Q, and Y236T cultures closely paralleled those of the reference strains and the Y236T variant when Ar versus N2 incubation also possess relatively high rates of acetylene reduction activities. These observations can be rationalized in terms of the Lowe and Thorneley model for nitrogenase catalysis (31, 32, 54), in which the enzyme must form a more highly reduced state (i.e., the so-called E3 or E4 state) to bind and reduce N2 than is required to reduce acetylene or protons (at the E3 or E2 state); i.e., certain mutations may lead to a less reduced cofactor.

Whereas wild-type Anabaena sp. strain PCC 7120 reduces acetylene only to ethylene, we noted the additional production of ethane in a number of our variants. Similarly, the wild-type MoFe protein of A. vinelandii produces only ethylene, but its Q191K, Q191E, H195N, and H195L derivatives produce 1.7 to differences between the A. vinelandii and Anabaena nitrogenases could affect their substrate reduction properties despite the high sequence conservation of the enzymes. Nevertheless, making such a comparison offers potential insights into the effects of the changes associated with the variant proteins.

Nitrogen-fixing capability. The ability to fix N2 was moderately or severely impaired in nearly all of the Anabaena NiFD variant strains examined. This result is consistent with expectations for the substitution of residues predicted to be proximal to the FeMo-co active site. Recent mutagenic, spectroscopic, and density functional theoretic studies have led to a prevailing view that the binding sites for N2, azide, acetylene, and hydrazine (N2H4) are located on the Fe2-Fe3-Fe6-Fe7 face of FeMo-co (7, 8, 12, 24, 29, 46). We observe that most modifications involving R284 and F388 (on the Fe2-Fe4-Fe5-Fe6 face) or Q193 and H197 (near Fe2 and Fe3, respectively) lead to substantial losses in N2 reduction activities. In contrast, the enzyme generally was more resilient to substitutions involving Y236 (near Fe1, Fe2, and Fe3) or S285 (near Fe1). Diminution of N2 reduction, as found in most of the mutants herein examined, is required if one aims to redistribute the six electrons involved in that process toward proton reduction.

Acetylene reduction. The nine Anabaena mutants capable of N2 fixation also possess relatively high rates of acetylene reduction. In addition, several variants that were severely impaired in N2 fixation retained significant acetylene and proton reduction activities. These observations can be rationalized in terms of the Lowe and Thornley model for nitrogenase catalysis (31, 32, 54), in which the enzyme must form a more highly reduced state (i.e., the so-called E3 or E4 state) to bind and reduce N2 than is required to reduce acetylene or protons (at the E3 or E2 state); i.e., certain mutations may lead to a less reduced cofactor.

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air versus Ar, ranging from 82 to 143% at 2 days after nitrogen step-down (Table 1), and indicate that N2 does not inhibit H2 production by these variant cultures for time periods of at least a week. Whereas the H2 accumulation rates of the R284H, H197Q, and Y236T cultures closely paralleled those of the reference strains under Ar during the first 4 days, the other NiFD variant cultures exhibited significantly decreased accumulation rates.

Quantification by CDW versus Chl a content. The H2, ethylene, and ethane production rates and H2 accumulation comparisons described above were all based on the assumption that the mutant strains contained comparable levels of Chl a regardless of the different incubation conditions. To test this hypothesis and to allow comparison with such measurements in other species, cultures incubated for 7 days under N2 + 5% CO2 or Ar + 5% CO2 were examined for both Chl a content and CDW (Table 2). The CDW measurements were very constant among the cultures, regardless of the incubation conditions. In contrast, the Chl a content varied nearly 2-fold for the reference strains and the Y236T variant when Ar versus N2 incubation was compared. Although for some cultures the results may lead to overestimation of the gas production rates or levels of H2 accumulation, we used cultures harvested at 1 day after nitrogen step-down, when the concentration of Chl a would be minimally affected.

DISCUSSION

We discuss the nitrogen fixation, acetylene reduction, and proton reduction activities of our Anabaena sp. strain PCC 7120 mutants in comparison to results on the very well-studied nitrogenase of A. vinelandii. Whereas we utilized intact cyanobacterial cells for our assays, the prior studies were carried out with soluble cell extracts or purified MoFe protein under conditions of optimal or sufficient amounts of Fe protein, Na2S2O4, and MgATP. One must be cautious when comparing results obtained from in vivo and in vitro studies; for example, the cellular level of MgATP is not readily controlled. Furthermore, we cannot rule out the possibility that subtle structural changes between the A. vinelandii and Anabaena nitrogenases could affect their substrate reduction properties despite the high sequence conservation of the enzymes. Nevertheless, making such a comparison offers potential insights into the effects of the changes associated with the variant proteins.

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TABLE 2. Cell dry weight, Chl a content, and Chl a/CDW ratios by selected variants after 7 days of incubation under Ar + 5% CO2 and N2 + 5% CO2.

| Strain     | CDW (mg) | Chl a (mg) | Chl a/CDW (mg/g) |
|------------|----------|------------|------------------|
|            | Under Ar | Under N2   | Under Ar         | Under N2         | Under Ar | Under N2         |
| ΔHup       | 0.1 2.4  | 0.1 2.4    | 0.1 2.4          | 0.1 2.4          | 0.1 2.4  | 0.1 2.4          |
| AnNiΔHup   | 0.1 2.7  | 0.1 2.7    | 0.1 2.7          | 0.1 2.7          | 0.1 2.7  | 0.1 2.7          |
| Q193B      | 0.1 3.0  | 0.1 3.0    | 0.1 3.0          | 0.1 3.0          | 0.1 3.0  | 0.1 3.0          |
| Q193H      | 0.1 3.1  | 0.1 3.1    | 0.1 3.1          | 0.1 3.1          | 0.1 3.1  | 0.1 3.1          |
| Q193L      | 0.1 3.2  | 0.1 3.2    | 0.1 3.2          | 0.1 3.2          | 0.1 3.2  | 0.1 3.2          |
| H197T      | 0.1 3.5  | 0.1 3.5    | 0.1 3.5          | 0.1 3.5          | 0.1 3.5  | 0.1 3.5          |
| H197F      | 0.1 3.6  | 0.1 3.6    | 0.1 3.6          | 0.1 3.6          | 0.1 3.6  | 0.1 3.6          |
| H197Q      | 0.1 3.7  | 0.1 3.7    | 0.1 3.7          | 0.1 3.7          | 0.1 3.7  | 0.1 3.7          |
| Y236T      | 0.1 3.8  | 0.1 3.8    | 0.1 3.8          | 0.1 3.8          | 0.1 3.8  | 0.1 3.8          |
| R284T      | 0.1 4.0  | 0.1 4.0    | 0.1 4.0          | 0.1 4.0          | 0.1 4.0  | 0.1 4.0          |
| R284H      | 0.1 4.1  | 0.1 4.1    | 0.1 4.1          | 0.1 4.1          | 0.1 4.1  | 0.1 4.1          |
| F388H      | 0.1 4.2  | 0.1 4.2    | 0.1 4.2          | 0.1 4.2          | 0.1 4.2  | 0.1 4.2          |

* Values are averages of two independent experiments, each performed with duplicate samples. The initial average cell dry weight (CDW), Chl a, and Chl a/CDW of all of the selected cultures at the starting times were 1.8 ± 0.1 mg, 30 µg, and 17 ± 1 µg/mg, respectively.
50% as much ethane as ethylene (17, 27, 44). The analogous *Anabaena* variants (Q193K, H197N, and H197L; Q193E was not examined) all produce ethane, as do many other variants. Most of these strains exhibited ethane/ethylene ratios of only ~0.1 to 10%, but the Q193K, H197T, and H197N strains exhibited ratios of 47, 17, and 24%, respectively. The H197T and H197N mutants were of special interest because they produced ethane rapidly. In general, substitutions of Q193 and H197 appear to modulate the reduction of acetylene to ethylene and ethane.

Ethane formation from acetylene is a characteristic of vanadium-containing (9) and Fe-only (37) nitrogenases. The ethane/ethylene ratios for these Mo-independent nitrogenases range from ca. 1% to several percent regardless of the microorganisms and strains (9, 26, 35, 37, 43). Compared to these alternative nitrogenases and most of the ethane-producing variants of Mo-nitrogenase, the Q193K and H197N variants reduce acetylene to ethane very effectively. Following the lead of Fisher et al. (17), we suggest that a longer residence time of the bound ethylenic intermediate at the active site might be responsible for the increased formation of ethane.

**H₂ production.** The rates of H₂ production by the Q193K, H197T, and R284H NifD *Anabaena* mutants under air approached or exceeded their rates under Ar (63 to 82%, 72 to 98%, and 99 to 120%, respectively), analogous to the *in vitro* results for the corresponding variants of *A. vinelandii*, where the O₂-labile activities were similar under N₂ and Ar (Q191K, H195T, and R277H, with ratios of 95, 77, and 99%, respectively) (27, 44, 48). In contrast, the cyanobacterial H197L and H197N NifD variants were much less effective at reducing protons in the presence of N₂ than in its absence (44 to 52% and 19 to 23%, respectively) relative to the relevant H195L and H195N *A. vinelandii* enzymes (115 and 95%, respectively) (27).

The *Anabaena* H197Q variant produced much less H₂ under air than under Ar (53%) much like the corresponding H195Q variant of *A. vinelandii*. The latter enzyme cannot reduce N₂ effectively, but N₂ can bind to the active site and inhibit proton reduction (10, 27). In addition, the binding of N₂ to that altered enzyme was shown to suppress total electron flux by uncoupling MgATP hydrolysis from electron transfer, as seen most strikingly under conditions of limiting MgATP (27). N₂ inhibition of H₂ production most likely explains the observed behavior for several other variants studied here. Moreover, the additional negative effects of suppressed electron flux as described for the H195Q *A. vinelandii* enzyme might account for the significantly lower values for the rate ratios (i.e., 10, 9, and 14%) of H₂ production in the presence of air versus Ar in the Q193V, Y236A, and S285N variants, respectively.

The H₂ production rates of three variants (Q193H, R284H, and F388H) under air or 100% N₂ (see Table S4 in the supplemental material) are similar to or slightly greater than those under Ar. This finding could indicate that N₂ interacts in a positive manner with the active site during proton reduction by these variant nitrogenases. A recent study showed that N₂, though not a substrate of the enzyme, increases H₂ production activity by reconstituted NifDK in which FeMo-co is replaced by NifB-co containing neither Mo nor homocitrate (50). The stimulatory effect of N₂ may relate to the N₂-dependent pathway for HD (hydrogen deuteride) formation in the presence of D₂ and N₂ (20, 30). Of interest, each of these highly active *Anabaena* variants is predicted to place an added His close to H197. The imidazole ring of the new residue could participate in His-mediated proton transfer to the FeMo-co site (13, 22, 51). In this regard, H195 of *A. vinelandii* nitrogenase, equivalent to the cyanobacterial H197, hydrogen bonds to the central bridging sulfur atom (S2B) between Fe2 and Fe6 of FeMo-co and was experimentally shown to be an obligate proton donor for nitrogenous substrates (N₂, azide, and hydrazine), but not for reduction of acetylene (3, 10, 27). We speculate that His residues replacing Q193, R284, and F388 in the *Anabaena* enzyme could hydrogen bond to neighboring residues, a water molecule, or homocitrate (in the case of Q193H) and participate in the transfer of protons to H197 in the active site or to homocitrate, which is proposed to be part of a water-filled channel potentially involved in proton transfer (13). In this regard, the Q193S variant, which also exhibits similar high H₂ production rates under Ar or air, could similarly participate in proton transfer via a hydrogen-bonded chain.

**Long-term accumulation of H₂.** Whereas certain *Anabaena* variants accumulated comparable (H197F, Y236T, and R284H) or less (eight other strains) H₂ than the reference strains during long-term incubations under Ar + 5% CO₂, the 11 selected *Anabaena* NifD variants (Fig. 4) greatly exceeded the H₂ accumulation of the reference strains under N₂ + 5% CO₂. Under these physiologically relevant conditions, the reference strains produced H₂ for only 2 to 3 days and only at low rates. This result can be rationalized in terms of the diazotrophic growth capability of these strains, where the nitrogen-sufficient nutritional status leads to decreased nitrogenase activity, as previously discussed (33). The Y236T variant, the only strain examined that exhibits some diazotrophic capability, ceased producing H₂ after 4 days of incubation under N₂, whereas all other of the 11 variants continued to accumulate H₂ over 1 week. In general, the sustained production of H₂ was comparable for most mutants whether incubated under Ar or N₂, although the amount of H₂ accumulated by the H197F and H197Q variants was only about half as great under N₂ (Fig. 4 and see Table S6 in the supplemental material).

The concentrations of H₂ accumulated for all of the cultures and conditions tested leveled off by about 1 week, an observation similar to those of previous reports (28, 57). Cessation of H₂ production is unlikely to be due to O₂ accumulation, which we measured as 6 to 9% under Ar or N₂ after 5 to 7 days (data not shown). Kumazawa and Mitsui (28) showed that H₂ production could be sustained for longer periods in flasks with periodic Ar gas replacement, resulting in increased cumulative H₂ production; however, the rates these researchers observed gradually decreased. Increasing levels of H₂ and O₂ in closed flasks results in increased pressure that was suggested to further inhibit H₂ and O₂ production (28). Another possibility is that H₂ production is balanced by H₂ uptake by the bidirectional hydrogenase (Hox) activity that was not deleted in these studies.

The R284H variant was most effective in accumulating H₂ under N₂ and therefore of greatest interest. The H₂ accumulation by this mutant under N₂ after 1 week was 87% of that observed for the two reference strains under Ar. This result highlights the potential of using this mutant as the parental strain for further mutagenesis studies in efforts to attain even greater levels of photobiological H₂ production.
Concluding comments. We demonstrate here the feasibility of engineering cyanobacterial strains for enhanced photobiological production of H₂ under aerobic growth conditions. In addition to further efforts to develop strains capable of even higher levels of H₂ production, many other hurdles will need to be overcome in order to develop this approach as a useful fuel source. For example, methods will need to be established for large-scale and long-term incubation of the cells under phototrophic H₂-producing conditions, as well as for capturing and storing the H₂ produced.

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