Expression of *Shewanella oneidensis* MR-1 [FeFe]-Hydrogenase Genes in *Anabaena* sp. Strain PCC 7120

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H₂ generated from renewable resources holds promise as an environmentally innocuous fuel that releases only energy and water when consumed. In biotechnology, photoautotrophic oxygenic diazotrophs could produce H₂ from water and sunlight using the cells’ endogenous nitrogenases. However, nitrogenases have low turnover numbers and require large amounts of ATP. [FeFe]-hydrogenases found in other organisms can have 1,000-fold higher turnover numbers and no specific requirement for ATP but are very O₂ sensitive. Certain filamentous cyanobacteria protect nitrogenase from O₂ by sequestering the enzyme within internally micro-oxic, differentiated cells called heterocysts. We heterologously expressed the [FeFe]-hydrogenase operon from *Shewanella oneidensis* MR-1 in *Anabaena* sp. strain PCC 7120 using the heterocyst-specific promoter P<sub>hetN</sub>. Active [FeFe]-hydrogenase was detected in and could be purified from aerobically grown *Anabaena* sp. strain PCC 7120, but only when the organism was grown under nitrate-depleted conditions that elicited heterocyst formation. These results suggest that the heterocysts protected the [FeFe]-hydrogenase against inactivation by O₂.

Liquefied H₂ is an attractive alternative to traditional fossil fuels because it has a very high energy content per unit weight (56), and when utilized as an energy source, H₂ releases water as the only by-product. Most of the H₂ currently utilized, however, is derived via steam reforming of natural gas or gasification of coal (47), and therefore, its formation still consumes fossil fuels. Many microorganisms produce H₂, often coupling the reduction of protons to the fermentation of reduced carbon (14, 59). H₂ generated by oxygenic photoautotrophs (e.g., microalgae and cyanobacteria) holds special promise because water is the substrate and the energy required for H₂ production is derived from light (33).

The two classes of enzymes that catalyze H₂ formation are nitrogenases and hydrogenases. Nitrogenases have relatively low turnover numbers, require 2 molecules of ATP for every electron used for reduction, and produce at least 1 mol of H₂ for every mole of N₂ reduced (58). In contrast, many hydrogenases have very high turnover numbers and utilize 100% of their electrons in proton reduction with no ATP requirement (29). Hydrogenases are classified according to the metals found at their active site: [NiFe]-hydrogenases are typically uptake, bidirectional, or H₂-sensing proteins; [Fe]-hydrogenases are involved in the pathway that converts CO₂ to CH₄ in methanogens; and [FeFe]-hydrogenases, although catalytically reversible, are typically involved in H₂ production (66). [FeFe]-hydrogenases are the most active H₂-forming enzymes known (29) and can have turnover numbers that are more than 1,000-fold greater than those characteristic of nitrogenases (34).

The deceptively simple equation 2H⁺ + 2e⁻ ⇌ H₂ belies the complexity of the reaction catalyzed by hydrogenases. Both electrons (28, 54) and protons (16) require pathways for transport to/from the active site, and multiple proteins are required to assemble the unusual active site. The proteins HydE, HydF, and HydG are responsible for the assembly and insertion of the di-iron subcluster at the active site of [FeFe]-hydrogenases, forming the complete six-iron prosthetic group (H cluster) and the catalytically active protein (50, 53).

One challenge in using oxygenic phototrophs for H₂ production is that [FeFe]-hydrogenases are rapidly and irreversibly inactivated by O₂ (31, 61, 67). This challenge can be overcome by separating photosynthesis and H₂ production either temporally or spatially. For example, many organisms separate photosynthesis and H₂ production by storing carbohydrates during the day and fermenting them at night when the cells become anaerobic (3, 11).

We have tested the strategy of separating photosynthesis and H₂ production spatially by using *Anabaena* sp. strain PCC 7120 as a host for heterologous expression of an [FeFe]-hydrogenase. That strain, sometimes called *Nostoc* sp. and referred to here as *Anabaena* sp., is a filamentous cyanobacterium that forms heterocysts, specialized cells that fix N₂ when the organism is deprived of fixed nitrogen (22, 26, 55). Nitrogenases, like [FeFe]-hydrogenases, are extremely sensitive to O₂ (52, 60). Heterocysts maintain a micro-oxic interior that protects nitrogenase from inactivation by O₂ by depositing a specialized cell envelope that limits entry of O₂ into the oxygen-evolving complex of photosystem II, and generating a highly active respiratory apparatus (49, 71). Because nitrogen fixation is a reductive process, heterocysts contain an electron transport system that shuttles electrons to nitrogenase via ferredoxin (5). Thus, an electron transport system suitable for [FeFe]-hydrogenases may already be present in heterocysts. By expressing an [FeFe]-hydrogenase and the maturation proteins needed for its activity specifically in the heterocysts of *Anabaena* sp., it may be possible to engineer an organism that can produce large amounts of H₂ even under fully aerobic conditions.
TABLE 1 Strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Derivation and/or relevant characteristics</th>
<th>Reference or source</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>Anabaena spp.</td>
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<tr>
<td>AMC414</td>
<td>Sm’ Sp’ Hup’</td>
<td>13</td>
</tr>
<tr>
<td>AMC414 DR3747</td>
<td>Sm’ Sp’ Nm’ Hup’ hoxY hoxH (double recombinant of pRL3747a)</td>
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<td>E. coli</td>
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<td>DH5α</td>
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<td>DH5αMCR</td>
<td>Received courtesy of J. C. Meeks (University of California, Davis)</td>
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<td>HB101</td>
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<td><strong>Plasmids</strong></td>
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<td>anp09016</td>
<td>Ap’; Anabaena sp. chromosomal DNA from bp 883713 to 889860 in the BamHI site of pUC18</td>
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<td>pDS4101</td>
<td>Ap’; ColK function enabling conjugal transfer</td>
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<td>pDU1</td>
<td>Nostoc sp. strain PCC 7524 plasmid used in chimeric vectors</td>
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<td>pET28a:hyd operon</td>
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<td>Ap’; cloning vector</td>
<td>Promega</td>
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<td>Cm’ Em’; pDU1; gluA promoter; the complete S. oneidensis MR-1 hyd operon was digested with NotI (blunted) and XbaI from the pET28 derivative and cloned into pRL2833a digested with Xhol (blunted) and AvrII</td>
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**MATERIALS AND METHODS**

**Bacterial strains, transfer of plasmids, and general growth conditions.**

Published procedures (20, 21, 73) were used for conjugal mobilization of plasmids from Escherichia coli into the Hup’ mutant AMC414 (13) of Anabaena sp. (the strains and plasmids used in this study are described in Table 1). Site-specific premethylation, nicking, and transfer of the DNA made use of bacterial strains E. coli HB101(pRL1124, pDS4101) and J53(RP4) or E. coli HB101(pRL623, pRL443). E. coli cells were grown at...
37°C in Luria-Bertani (LB) medium containing antibiotics (25 or 30 μg ml$^{-1}$ chloramphenicol [Cm], 100 μg ml$^{-1}$ ampicillin [Ap], 50 μg ml$^{-1}$ kanamycin [Km], and/or 50 μg ml$^{-1}$ streptomycin sulfate [Sm]), as appropriate. Cultures of AMC414 and its derivatives were grown in an 8-fold dilution of nitrate-free Allen and Arnon (AA) liquid growth medium (AA/8) (39) under continuous shaking or on full-strength AA solid medium (39) at 30°C with continuous illumination (30 μmol photons m$^{-2}$ s$^{-1}$ of photosynthetically active radiation). Where indicated, liquid media were supplemented with 2.5 mM KNO$_3$ and 2.5 mM NaNO$_3$. Cultures of AMC414 were supplemented with 4 μg spectinomycin dihydrochloride pentahydrate (Sp) ml$^{-1}$, and cultures of derivatives of that strain were also supplemented with erythromycin (Em; 5 μg ml$^{-1}$ in liquid medium, 10 μg ml$^{-1}$ in agar) and/or neomycin (Nm; 20 μg ml$^{-1}$ in liquid medium, 30 μg ml$^{-1}$ in agar).

Expression of [FeFe]-hydrogenase in _E. coli_. _E. coli_ DH5α containing plasmid pKG3 or pKG6 was grown overnight in LB medium supplemented with Cm. A 500-ml culture inoculated with 1 ml of the overnight suspension was grown until it reached an optical density at 600 nm of 0.1 to 0.3. After incubation for 3 days at 30°C with the culture continuously bubbled with air, the cells were again harvested by centrifugation, the supernatant was decanted, and the pellet was brought into the Coy chamber. To remove the cell pellet residual O$_2$ that was not eliminated by the vacuum cycles during entry into the Coy chamber, the pellet was resuspended in 7 ml of anoxic AA/8 using 7 Eppendorf tubes, sedimented, and decanted. Dilution, sedimentation, and decantation were repeated a second time. Finally, the cells in each Eppendorf tube were resuspended in 1 ml of Ni-NTA buffer containing 10 mM dithionite. The cells were again harvested by centrifugation, the supernatant was decanted, and the pellet was brought into the Coy chamber. To remove the cell pellet residual O$_2$ that was not eliminated by the vacuum cycles during entry into the Coy chamber, the pellet was resuspended in 7 ml of anoxic AA/8 using 7 Eppendorf tubes, sedimented, and decanted. Dilution, sedimentation, and decantation were repeated a second time. Finally, the cells in each Eppendorf tube were resuspended in 1 ml of Ni-NTA buffer containing 10 mM dithionite. The cells were then sonicated, and [FeFe]-hydrogenase was purified as described above.

**Preparation of _Anabaena_ cells for purification of hydrogenase protein.** Five 400-ml cultures of nitrate-deprived AMC414 (pKG13) were harvested by centrifugation, and the five cultures were combined and concentrated to 30 ml. The cells were transferred to a 125-ml Erlenmeyer flask, and the flask was sealed with a rubber septum. Except for the few instances noted below, the combined cultures were inoculated in the light with shaking for 2 h while being bubbled with an Ar-N$_2$-CO$_2$ (79%, 20%, 1%) mixture using two needles (one for entry of the gas mixture and one to release the pressure) to diminish the concentration of dissolved O$_2$ in the growth medium. The cells were then transferred to airtight centrifuge vials (Nalgene) in an anaerobic Coy chamber and sedimented by centrifugation (10 min at 17,000 × g). The supernatant was decanted in the Coy chamber, and the cells were resuspended in 7 ml of anoxic Ni-NTA buffer containing 10 mM dithionite. Five 400-ml cultures of AMC414 without the pKG13 plasmid were treated similarly. The supernatant solutions were then treated as described above for the purification of [FeFe]-hydrogenase from _E. coli_.

When the cultures of AMC414 and AMC414 (pKG13) were not sparged with the Ar-N$_2$-CO$_2$ gas mixture prior to purification of the hydrogenase, the aerobically grown cells were harvested, resuspended in 30 ml as described above, and then bubbled with air for an additional 1 h. The cells were again harvested by centrifugation, the supernatant was decanted, and the pellet was brought into the Coy chamber. To remove from the cell pellet residual O$_2$ that was not eliminated by the vacuum cycles during entry into the Coy chamber, the pellet was resuspended in 7 ml of anoxic AA/8 using 7 Eppendorf tubes, sedimented, and decanted. Dilution, sedimentation, and decantation were repeated a second time. Finally, the cells in each Eppendorf tube were resuspended in 1 ml of Ni-NTA buffer containing 10 mM dithionite. The cells were then sonicated, and [FeFe]-hydrogenase was purified as described above.

**In vitro assay of H$_2$ evolution.** Supelco 10-ml serum vials (Sigma-Aldrich, St. Louis, MO) were filled with either 4.5 ml (_Anabaena_ experiments) or 1.5 ml ( _E. coli_ experiments) of Ar-sparged H$_2$-evolution buffer (50 mM HEPES [pH 7.0], 500 mM NaCl, 100 mM Na$_2$S$_2$O$_4$, 10 mM methyl viologen [MV]) and sealed with butyl rubber septa (Bellco Glass, Vineland, NJ). The headspace gas was exchanged with Ar using standard Schlenk line techniques (9). Purified hydrogenase (0.5 ml) was injected into the vial using a gas-tight syringe, and H$_2$ accumulation was measured over time by injecting 50 μl of the headspace gas onto a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific) equipped with a thermal conductivity detector (TCD) and a capillary molecular sieve column (30 m; inner diameter, 0.53 mm; RT-Msieve 5A; Restek Corp., State College, PA), using Ar as the carrier gas (10 ml min$^{-1}$, 70°C). H$_2$ was detected by a TCD in which the block temperature was 150°C, the transfer temperature was 130°C, and the filament temperature was 350°C. Samples were injected every 10 to 20 min until five measurements were taken, and then a final measurement was made approximately 12 h later. During the reaction, the serum vials were inverted to minimize gas diffusion and incubated at 25°C with shaking. A vial containing only H$_2$ evolution buffer and 0.5 ml of purification buffer (Ni-NTA buffer containing 100 mM imidazole) in place of purified [FeFe]-hydrogenase served as a negative control, and a new standard curve was generated daily by injecting increasing amounts of a mixture of 0.5% H$_2$ in Ar.

**In situ assays of H$_2$ evolution.** Cells were grown and washed as described for heterocyst induction. After 2 days of nitrate deprivation, a 200-ml volume of cell culture was harvested and concentrated about 7-fold, yielding 5 to 10 μg Chl a ml$^{-1}$. Concentrated cultures were transferred to serum vials (2 ml of culture in a 10-ml vial). After the vials were evacuated and filled with Ar, 20 μl of an anoxic stock solution (1 M sodium dithionite, 0.5 M MV, 2 M TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; pH 7.6], 0.25% Triton X-100) was added to each vial to provide reductant for hydrogenase. The small amount of the detergent Triton X-100 was added to facilitate entry of the electron donor into the heterocysts. In the presence of dithionite and the absence of ATP and an ATP-generating system, nitrogenase is inactive (8, 62). Samples

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were incubated with shaking in the light (70 μmol photons m⁻² s⁻¹) for 2 to 3 h. The H₂ in the headspace was quantified by injecting 200 μl of headspace gas into a gas chromatograph as described above.

Western blot analysis. For Western blot analysis, 12.5 μl of purified protein was mixed with an equal volume of 2x SDS-PAGE loading dye (100 mM Tris-HCl [pH 6.8], 4.0% electrophoresis-grade SDS, 0.2% bromophenol blue, 20.0% glycerol, 20.0 mM dithiothreitol) and denatured at 65°C for 15 min. The samples were loaded onto a 12% running gel and subjected to electrophoresis at 120 V for approximately 100 min. The proteins were transferred to a polyvinylidene difluoride membrane (Merck, EMD Millipore, Darmstadt, Germany) at 60 V for approximately 2.5 h according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). After the blotting procedure, the membrane was placed in 10 ml of a 5% solution of bovine serum albumin in Tris-buffered saline-Tween 20 (TBST) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) and incubated for 1 h. The blot was washed 3 times at room temperature with 10 ml TBST buffer and probed overnight according to the manufacturer’s instructions (Novagen, EMD Millipore) using a mouse antibody against the 6x His epitope tag as the primary probe. The secondary antibody was a goat anti-mouse antibody fused to horseradish peroxidase (Pierce, Thermo Fisher Scientific Inc., Rockford, IL). The conjugated antibody was detected using a SuperSignal West Pico kit (Thermo Fisher Scientific) according to the manufacturer’s instructions and was imaged with a Fujifilm LAS-3000 camera (Fujifilm Holdings Corp., Tokyo, Japan).

RESULTS

Design and characterization of the plasmids used for heterologous expression. The proper assembly of [FeFe]-hydrogenases requires a series of maturation proteins for the biosynthesis and insertion of the di-iron subcluster into the active site (41, 45, 51). Expression of large quantities of functional [FeFe]-hydrogenase therefore requires the coordinated regulation and expression of multiple genes to ensure the correct ratio between the different gene products. Our strategy for ensuring proper protein ratios was to use a natural operon, where all of the hydrogenase genes are encoded in one transcriptional unit. We chose the [FeFe]-hydrogenase operon from the bacterium Shewanella oneidensis MR-1 (Fig. 1A), an operon that contains all five hydrogenase-related genes (hydA, hydB, hydE, hydF, and hydG) as well as two additional genes of unknown function (one annotated as a putative formate dehydrogenase) (36). The operon was modified such that the large hydrogenase subunit (HydA) harbored an N-terminal 6x His epitope tag for subsequent purification. In S. oneidensis MR-1, the [FeFe]-hydrogenase is transferred to the periplasm. Our analysis revealed a twin-arginine-translocation (TAT) signal peptide at the N terminus of the small hydrogenase subunit (HydB), a peptide that is presumably cleaved either during or after the transport of the hydrogenase to the periplasm. Anabaena sp. contains the TAT machinery (17), and it therefore seemed likely that the native S. oneidensis MR-1 [FeFe]-hydrogenase would be transferred to the periplasm when heterologously expressed in strain AMC414. Because reductant might not reach the periplasm of the heterocysts and under aerobic conditions the periplasm of Anabaena sp. heterocysts might have a higher O₂...
activated irreversibly by O2, we typically sparged the medium was used to wash and resuspend one of the cell cultures. The [FeFe]-hydrogenase was purified from the strain expressing pKg6 had a slightly higher specific activity than the hydrogenase purified from the strain expressing the unmodified operon in pKG3, demonstrating that the deletion of the TAT signal peptide at the predicted cleavage site (Fig. 1A) and inserting a new N terminus.

To test whether hydrogenase activity might be affected by this modification, we separately expressed each version of the operon in either pKG3 (with TAT) or pKG6 (without TAT) in E. coli DH5a under anaerobic conditions. The [FeFe]-hydrogenase was purified from each resulting strain and tested for activity. As shown in Fig. 1B, the [FeFe]-hydrogenase purified from strain AMC414(pKG13) cells with and without sparging with Ar-CO2-N2 for 2 h to 3 days. In vitro H2 evolution assays (Fig. 2A) demonstrated that the hydrogenase purified from the AMC414(pKG13) cell extract was active. Virtually no H2 was detected from identically processed protein extract from AMC414 lacking the plasmid, indicating that the S. oneidensis MR-1 [FeFe]-hydrogenase expressed in AMC414 was responsible for the H2 evolution observed. Results of Western blotting assays confirmed that HydA, the large subunit of hydrogenase, was produced in AMC414(pKG13) cells (Fig. 3A).

Under our growth conditions and in the presence of nitrate, Anabaena sp. cultures contain very few heterocysts (32). To test whether [FeFe]-hydrogenase was expressed and active in heterocysts of AMC414(pKG13), we compared the results of using AMC414(pKG13) cells that were deprived of nitrate for 2 to 3 days. In vitro H2 evolution assays (Fig. 2A) demonstrated that the hydrogenase purified from the strain AMC414(pKG13) cells that were not sparged with Ar, verifying the presence of active [FeFe]-hydrogenase in filaments of AMC414(pKG13) grown aerobically. The bar graphs represent the mean relative hydrogenase activities and standard deviations of two independent experiments. (B) Comparison of in vitro activities of [FeFe]-hydrogenase purified from AMC414(pKG13) cells grown in the presence or absence of nitrate. Activity was observed only following nitrate deprivation. The bar graphs represent the mean relative hydrogenase activities and standard deviations of three independent experiments. (C) Comparison of in vitro activities of hydrogenase purified from AMC414(pKG13) cells with and without sparging with Ar-CO2-N2 for 2 h immediately prior to cell harvesting. Significant quantities of H2 were produced by [FeFe]-hydrogenase purified from cells that were not sparged with Ar, verifying the presence of active [FeFe]-hydrogenase in filaments of AMC414(pKG13) grown aerobically. The bar graphs represent the mean relative hydrogenase activities and standard deviations of two independent experiments.

FIG 2 In vitro activity of [FeFe]-hydrogenase heterologously expressed in Anabaena sp. The majority of the active hydrogenase typically eluted in fractions 2 and 3 from the affinity column. The results from the most active elution fraction and the corresponding buffer controls are shown. In all cases, the activity of the protein purified from AMC414 cells (Hup−) harboring pKG13 deprived of nitrate was normalized to 1, and therefore, it has no standard deviation. (A) AMC414 with and without plasmid pKG13. The fractions obtained from AMC414 lacking pKG13 yielded essentially no hydrogenase activity. The bar graphs represent the mean relative hydrogenase activities and standard deviations of two independent experiments. (B) Comparison of in vitro activities of [FeFe]-hydrogenase purified from AMC414(pKG13) cells grown in the presence or absence of nitrate. Activity was observed only following nitrate deprivation. The bar graphs represent the mean relative hydrogenase activities and standard deviations of three independent experiments. (C) Comparison of in vitro activities of hydrogenase purified from AMC414(pKG13) cells with and without sparging with Ar-CO2-N2 for 2 h immediately prior to cell harvesting. Significant quantities of H2 were produced by [FeFe]-hydrogenase purified from cells that were not sparged with Ar, verifying the presence of active [FeFe]-hydrogenase in filaments of AMC414(pKG13) grown aerobically. The bar graphs represent the mean relative hydrogenase activities and standard deviations of two independent experiments.
sparged with air prior to purification under anoxic conditions (Fig. 3B). Furthermore, in vitro activity assays showed that [FeFe]-hydrogenase activity was largely insensitive to whether or not the cell culture was sparged with Ar-CO₂-N₂ (Fig. 2C).

**In situ expression in Anabaena.** The activity of [FeFe]-hydrogenase expressed heterologously in *Anabaena* sp. was also tested in situ in AMC414 DR3747, a strain lacking both uptake (Hup⁻) and bidirectional (Hox⁻) [NiFe]-hydrogenase activity. This strain was chosen to minimize H₂ uptake. Under conditions that facilitate electron transport to the [FeFe]-hydrogenase and are known to leave nitrogenase inactive (8, 62), AMC414 DR3747 lacking the [FeFe]-hydrogenase operon (without pKG13) produced virtually no H₂ (Fig. 4). However, when AMC414 DR3747 bearing the [FeFe]-hydrogenase-encoding plasmid pKG13 was provided with sodium dithionite and MV, we reproducibly observed formation of H₂ by [FeFe]-hydrogenase at about half the rate that was shown by the native, intact Hox protein when nitrogenase was inactive (AMC414 cells lacking pKG13).

**DISCUSSION**

Tsyrkanskov et al. (64) observed that a solar bioreactor approximatively 1/4 m² in area containing *Anabaena variabilis* ATCC 29413, a close relative of *Anabaena* sp., generated up to 1.1 liters of H₂ per day utilizing its native nitrogenase. How might H₂ production in *Anabaena* sp. be further increased? One strategy that has been pursued is to modify nitrogenase (44, 70), but thus far these efforts have not succeeded in increasing maximal production of H₂ by nitrogenase. A second strategy is to utilize a different H₂-producing enzyme. Because some [FeFe]-hydrogenases have turnover numbers 1,000-fold greater than those of nitrogenases (34), this approach has the potential to improve H₂ production rates significantly.

We therefore genetically engineered *Anabaena* sp. to express an [FeFe]-hydrogenase specifically in heterocysts. Although heterocysts lack an O₂-producing photosystem II, they do contain a functional photosystem I that can be a source of low-potential electrons. We employed a modified version of the *hyd* operon from *S. oneidensis* MR-1 and deleted the TAT signal at the N terminus of HydB to prevent shutting of the mature enzyme into the periplasm of the heterocysts. Our rationales for doing so were that the periplasm may contain a higher O₂ concentration than the cytosol and that photosystem I-reduced ferredoxin might be unable to access the [FeFe]-hydrogenase to reduce it. By deleting the TAT signal at the predicted cleavage site, we generated an enzyme that remained in the cytosol yet should be essentially identical to the mature enzyme in *S. oneidensis*. Because the [FeFe]-hydrogenase active site is assembled prior to TAT-mediated transport, there was no need to relocate the maturation proteins HydE, HydF, and HydG.

The *S. oneidensis* MR-1 *hyd* operon driven by the *hetN* promoter and lacking the TAT signal on HydB generates active hydrogenase when expressed in *Anabaena* sp. Both *in vitro* (Fig. 2) and *in situ* (Fig. 4) assays demonstrated that the heterologously expressed [FeFe]-hydrogenase produced H₂ when supplied with sodium dithionite and MV. As predicted, this activity was observed only after we deprived the cells of nitrate in the growth medium (Fig. 2B), suggesting that the proteins needed for H₂ production by the *S. oneidensis* MR-1 [FeFe]-hydrogenase were expressed in the *Anabaena* sp. heterocysts.

Using *Synechococcus elongatus* strain PCC 7942, a unicellular non-nitrogen-fixing cyanobacterium, Ducat et al. observed an *in vivo* H₂ evolution activity of 2.8 μmol H₂ h⁻¹ mg Chl a⁻¹ when expressing the *Clostridium acetobutylicum* [FeFe]-hydrogenase under anaerobic conditions (18). This activity is close to the *in situ* activity that we observed from the heterologously expressed [FeFe]-hydrogenase in our engineered strain [AMC414 DR3747(pKG13)]. However, our *in situ* H₂ evolution activity is only about 20% of that obtained from the endogenous nitrogenase (44), despite the theoretically much higher turnover number of [FeFe]-hydrogenases. Higher activities have been reported for the Hup⁺, unicellular cyanobacterium *Cyanothecce* sp. strain ATCC 51142 under Ar in the light (48). Surprisingly, even higher activities under aerobic conditions were reported (2).

How might the *in vivo* [FeFe]-hydrogenase activity in *Anabaena* sp. be increased? Küchenreuther et al. demonstrated that increasing FeS cluster production enhanced [FeFe]-hydroge-

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**FIG 3** (A) Western blot analysis showing the presence of HydA in AMC414(pKG13) cells but not in AMC, i.e., AMC414 cells lacking pKG13. (B) Western blot analysis demonstrating the presence of HydA in AMC414(pKG13) cultures that had been sparged either with air or with Ar-CO₂-N₂ for 2 h immediately prior to cell harvesting.

**FIG 4** Rate of H₂ production in whole filaments of AMC414 and AMC414 DR3747, with and without heterologous expression of *S. oneidensis* MR-1 *hyd* genes (carried on pKG13). AMC414 lacks Hup activity, and AMC414 DR3747 lacks both Hup and Hox activity. Nitrogenase is inactive under the experimental conditions used in this assay. Mean activities and standard deviations of 4 replicates in each of 3 independent experiments are shown.
nase maturation in *E. coli* (42), and a similar strategy could be employed with *Anabaena* sp. Another potential strategy for increasing H₂ production is to improve the efficiency of electron transfer between electron donors and hydrogenase in the heterocyst. Because heterocysts contain a ferredoxin linked to their photosynthetic electron transport chain, we had anticipated rapid electron transfer to the heterologously expressed [FeFe]-hydrogenase. However, different [FeFe]-hydrogenases interact to various degrees with different ferredoxins (1, 27), and the plant-like ferredoxin in *Anabaena* (5) may not interact efficiently with the bacterial hydrogenase used in this study. Ducat et al. noted that the in vivo activity of the *C. acetybuthylicum* [FeFe]-hydrogenase expressed in *S. elongatus* increased approximately 2-fold when a suitable ferredoxin was coexpressed (18), and a similar approach of coexpressing an additional ferredoxin might be advantageous in *Anabaena* sp. However, Agapakis et al. observed very low hydrogenase activity with *S. oneidensis* MR-1 [FeFe]-hydrogenase and different ferredoxins compared to other combinations (1). In addition, there is evidence that some dimeric [FeFe]-hydrogenases receive their electrons from cytochrome c instead of ferredoxin (19, 46). The *S. oneidensis* MR-1 [FeFe]-hydrogenase is dimeric, and there are abundant cytochromes in both the periplasm and the outer membranes of *S. oneidensis* MR-1 (7, 57). If *S. oneidensis* MR-1 [FeFe]-hydrogenase accepts electrons preferentially from cytochrome c, it could account for our ability to detect hydrogenase activity only in vitro and in situ with dithionite and not in vivo. Therefore, the expression of a different [FeFe]-hydrogenase might greatly increase in vivo H₂ production in *Anabaena* sp.

The principal rationale for expressing an [FeFe]-hydrogenase in heterocysts is to be able to produce abundant H₂ even under oxic conditions that would normally inactivate an O₂-sensitive hydrogenase. It has been proposed that O₂ enters heterocysts principally via the pores at the ends of those cells where there is no glycolipid layer to impede its entry (68) and that the so-called honeycomb membranes near the heterocyst pores are sites of respiratory complexes that reduce O₂ to water (71). Dinitrogenase reductase may further deplete O₂ from the interior of the heterocyst (63). The resulting micro-oxic environment (23, 37, 75) enables heterocysts to maintain nitrogen-fixing activity even in an oxic atmosphere. Active hydrogenase was purified from *Anabaena* sp. expressing the [FeFe]-hydrogenase operon even when the cultures were continuously sparged with air (Fig. 2C), suggesting that heterocysts also effectively protect [FeFe]-hydrogenase from inactivation by O₂, validating our approach.

In summary, we have successfully expressed an [FeFe]-hydrogenase in the heterocysts of *Anabaena* sp. The hydrogenase was active in situ and could be purified using affinity chromatography. Essentially the same amount of active [FeFe]-hydrogenase was purified regardless of whether the *Anabaena* sp. cultures were sparged with Ar-N₂-CO₂ or air. These results support the feasibility of using hydrogenases in heterocyst-forming cyanobacteria as a means to generate H₂ from sunlight and water under oxic conditions.

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