Heterologous Expression of Alteromonas macleodii and Thiocapsa roseopersicina [NiFe] Hydrogenases in Synechococcus elongatus

Philip D. Weyman¹, Walter A. Vargas¹✉, Yingkai Tong¹, Jianping Yu², Pin-Ching Maness², Hamilton O. Smith¹, Qing Xu¹✉

¹ Department of Synthetic Biology and Bioenergy, The J. Craig Venter Institute, Rockville, Maryland, United States of America, ²National Renewable Energy Laboratory, Golden, Colorado, United States of America

Abstract

Oxygen-tolerant [NiFe] hydrogenases may be used in future photobiological hydrogen production systems once the enzymes can be heterologously expressed in host organisms of interest. To achieve heterologous expression of [NiFe] hydrogenases in cyanobacteria, the two hydrogenase structural genes from Alteromonas macleodii Deep ecotype (AltDE), hynS and hynL, along with the surrounding genes in the gene operon of HynSL were cloned in a vector with an IPTG-inducible promoter and introduced into Synechococcus elongatus PCC7942. The hydrogenase protein was expressed at the correct size upon induction with IPTG. The heterologously-expressed HynSL hydrogenase was active when tested by in vitro H₂ evolution assay, indicating the correct assembly of the catalytic center in the cyanobacterial host. Using a similar expression system, the hydrogenase structural genes from Thiocapsa roseopersicina (hynSL) and the entire set of known accessory genes were transferred to S. elongatus. A protein of the correct size was expressed but had no activity. However, when the 11 accessory genes from AltDE were co-expressed with hynSL, the T. roseopersicina hydrogenase was found to be active by in vitro assay. This is the first report of active, heterologously-expressed [NiFe] hydrogenases in cyanobacteria.

Introduction

Hydrogen (H₂) production from photosynthetic microorganisms is an attractive strategy to store solar energy as a fuel [1]. H₂ fuel cells can provide carbon-free power; however, most H₂ currently in use derives from fossil fuels [1]. Development of photobiological H₂ production using photosynthetic microorganisms such as cyanobacteria and micro-algae can provide an alternative to fossil fuels by using the energy of the sun to convert H₂O into H₂.

H₂ can be produced by cyanobacteria using either nitrogenase or hydrogenase enzymes [2]. Hydrogenases catalyze the reversible reduction of protons to H₂ and can be divided into three phylogenetically-distinct categories that correspond with the metal composition of the active site: [FeFe], [NiFe], and the [Fe] hydrogenases of methanogens [3,4]. Two different groups of [NiFe] hydrogenases, the uptake hydrogenases and the bidirectional hydrogenases, have been found in many cyanobacterial genomes [2]. The uptake hydrogenases in cyanobacteria function largely in recycling H₂ produced as a byproduct of nitrogen fixation while bidirectional hydrogenases have been implicated in disposing of excess reductant as H₂ [5,6].

H₂ production from photosynthetic microorganisms such as cyanobacteria requires hydrogenases to be tolerant of oxygen produced from photosynthesis if H₂ production is to occur during daytime. Of the major categories of hydrogenases, [FeFe] hydrogenases are the most O₂-sensitive and are irreversibly destroyed by exposure to oxygen [7]. Most [NiFe] hydrogenases are temporarily inactivated by O₂ but can be reactivated upon returning to anaerobic conditions given sufficient reducing conditions [7]. All cyanobacterial [NiFe] hydrogenases studied thus far are sensitive to O₂ and function only briefly in aerobic conditions before being inactivated [8]. Nonetheless, several [NiFe] hydrogenases from other microorganisms maintain activity in the presence of oxygen, including those from Ralstonia eutropha [9], Rubrivivax gelatinosus [10], and Alteromonas macleodii [11].

Using O₂-tolerant hydrogenases in future cyanobacterial hydrogen production systems will require their heterologous expression in cyanobacteria, and expression is currently a barrier to the wide-spread use of foreign hydrogenases in cyanobacteria. The catalytic core of [NiFe] hydrogenases generally consists of two subunits, one large (ca. 60 kDa) and one small (ca. 30 kDa). The large subunit contains the [NiFe] catalytic site and requires an extensive set of accessory proteins to assemble an active catalytic site [12]. Maturation of the small subunit is not as well understood, but some [NiFe] hydrogenases require specific accessory proteins to assist in this process [13]. The accessory proteins are usually...
specific for the hydrogenase with which they have co-evolved, but may be active on closely related hydrogenases from another species [14, 15].

The complete and clustered set of accessory genes from AltDE may simplify the task of heterologous expression [16]; however, other organisms, including Thiothrix roseopersicina that contains a related [NiFe] hydrogenase, have accessory genes distributed throughout the genome [17]. Although the T. roseopersicina genome has not been sequenced, mutational analysis has identified several accessory genes (hynD, hupCDHIK and hypC1C2DEF) [17, 18, 19]. Here, we report the heterologous expression of active [NiFe] hydrogenases from AltDE and T. roseopersicina in the cyanobacterium Synechococcus elongatus PCC7942. Heterologous expression of an active [NiFe] hydrogenase has not been reported previously in cyanobacteria, and the development of systems for heterologous expression of hydrogenases in cyanobacteria may open up new possibilities for photobiological hydrogen production.

Results

Construction of the hoxYH hydrogenase mutant in S. elongatus PCC7942

The genome of S. elongatus PCC7942 contains the hoxYH genes encoding one [NiFe] bidirectional hydrogenase (HoxYH) (http://genome.jgi-psf.org/synel/synel.home.html). To eliminate background hydrogenase activity in S. elongatus to better detect the activity from heterologously-expressed enzymes, we knocked out the endogenous hydrogenase by transforming with a plasmid (pPW416) that would replace the hoxYH genes with an antibiotic cassette via DNA recombination. Complete segregation of the knockout mutant, named PW416, lacked any detectable hydrogenase activity (Table S2).

Expression of AltDE HynSL hydrogenase in S. elongatus PCC7942

AltDE contains one [NiFe] hydrogenase, HynSL, which has been characterized as oxygen-tolerant and thermostable [11]. The structural genes, hynSL, are surrounded by 11 genes encoding accessory proteins involved in the assembly and maturation of the [NiFe] hydrogenase catalytic site and the hydrogenase complex. A subset of these genes has been determined to play a critical role in hydrogenase maturation [16]. In order to express HynSL in S. elongatus, plasmid pRC41 was constructed by cloning the hydrogenase structural genes, hynSL, and the 11 adjacent genes into an expression plasmid [16] (Fig. 1). This plasmid contains a copy of the lacI gene [20] and allows for expression of the AltDE hydrogenase operon from the IPTG-inducible PTrc promoter. The plasmid pRC41 also contains flanking sequence for insertion of the hydrogenase gene cluster into “neutral site 1” (NSI) of the S. elongatus chromosome via homologous DNA recombination [21].

The construct was introduced into the S. elongatus hydrogenase knockout strain, PW416, to create strain RC41.

To examine the expression of HynSL, RC41 cultures expressing the AltDE hydrogenase gene operon were grown and induced with IPTG, and protein extracts from lysed cells were separated on an SDS-PAGE gel for immunoblotting. Western blotting was performed with antiserum raised against the T. roseopersicina [NiFe] hydrogenase large subunit, HynL. When induced with IPTG, a single band corresponding to the mature form of the AltDE, HynL (67 kDa) was detected (Fig. 2A). Without IPTG induction, no HynL band was detected. As a control, a duplicate gel was stained with Coomassie blue to confirm equal loading in each lane (Fig. 2B); IPTG was added to a final concentration of 5, 20, 100, or 200 μM to RC41 cultures to determine the optimal concentration of IPTG for protein expression. Western blotting analysis indicated that 100 μM IPTG yielded maximal expression of HynL (data not shown) and this concentration of IPTG was used for all future experiments.

To determine whether the AltDE HynSL protein was expressed with an active catalytic site in S. elongatus PCC7942, in vitro H2 evolution assays were performed with protein extracts from RC41. As expected, no hydrogenase activity was detected in the PW416 (ΔhoxYH) strain (Fig. 2C). Hydrogenase activity was detected in strain RC41 expressing both hydrogenase and accessory proteins from AltDE, and activity was strongly induced by IPTG. The activity from the heterologously expressed hydrogenase in RC41 represented one tenth of the native activity in the wild type S. elongatus strain (Fig. 2C). A small amount of activity was detected in the absence of IPTG induction, indicating slightly leaky expression from the PTrc promoter.

Expression of T. roseopersicina HynSL hydrogenase in S. elongatus PCC7942

In addition to expressing AltDE HynSL, we expressed its related stable hydrogenase, HynSL, from T. roseopersicina in S. elongatus. We assembled the sequences of T. roseopersicina accessory genes into two sets of plasmids. In the first set of plasmids, pHyn4-NSII, hynD (encoding the protease) as well as hupK and hypC1C2DEF were assembled along with the hydrogenase structural genes hynSL in an IPTG-inducible vector (Fig. 3), which could integrate into the

Figure 1. Diagram of pRC41 used for expression of AltDE hydrogenase HynSL in S. elongatus. Arrows indicate the direction of open reading frames. The figure was drawn to scale.

doi:10.1371/journal.pone.0020126.g001
“neutral site II” (NSII) of _S. elongatus_ through homologous recombination [21]. The plasmid was introduced into the hydrogenase knockout mutant (PW416) to make strain Hyn4. We further modified plasmid pHyn4-NSII to include two additional genes, _isp1_ and _isp2_. These two genes are found between _hynS_ and _hynL_ in the _T. roseopersicina_ chromosome [22]. They were removed in the construction of pHyn4-NSII but were added back downstream of the _hypDEF_ genes in the construction of pHyn5-NSII (Fig. 3). _Isp1_ and _Isp2_ are not predicted to play a role in hydrogenase maturation but rather confer electron transfer necessary for in vivo hydrogenase activity [23]. Plasmid pHyn5 was mobilized into the hydrogenase knockout strain to make Hyn5.

In the second set of plasmids, pHup-NSI, the _T. roseopersicina_ accessory genes _hupCDHI_ and an ORF of unknown function were assembled in an IPTG-inducible vector, which could recombine at the _S. elongatus_ NSI locus (Fig. 3). After mobilization of pHup-NSI into the hydrogenase knockout mutant, PW416, the resulting strain was named Hup. The pHup-NSI plasmid was also mobilized into Hyn4 and Hyn5 to create strains Hyn4/Hup and Hyn5/Hup, respectively.

_S. elongatus_ cell cultures expressing different combinations of _T. roseopersicina_ structural and accessory genes were induced with IPTG, and protein extracts prepared from lysed cells were analyzed by immunoblot after SDS-PAGE electrophoresis using anti-HynL antisera. A band corresponding to the correct size of the mature form of HynL was detected (62 kDa), and increased expression was observed in the presence of IPTG (Fig. 4). Similar levels of HynL expression were observed for all strains that contained HynSL (Fig. 4). In vitro hydrogen evolution assays were performed to determine whether the expressed hydrogenase possessed activity. No activity was detected in strains Hyn4, Hyn5, Hyn4/Hup or Hyn5/Hup (data not shown).

**Expression of the _T. roseopersicina_ HoxYH in _S. elongatus_**

Since the _T. roseopersicina_ HynSL hydrogenase was not properly assembled into its active form in _S. elongatus_ using the native cyanobacterial accessory proteins, we sought to determine whether an enzyme with greater similarity to the cyanobacterial hydrogenase such as HoxYH might be successfully assembled and possess activity when heterologously expressed. _T. roseopersicina_ also contains a bidirectional hydrogenase encoded by genes _hoxHII_, which has been shown to interact with the diaphorase subunit encoded by genes _hoxEFU_ [23]. The small and large subunits are 44 and 54 percent similar, respectively, to the native bidirectional hydrogenase in _S. elongatus_ [24]. Given the similarities between the two proteins, a functional _T. roseopersicina_ HoxYH may be able to be assembled and processed by the native _S. elongatus_ accessory proteins. To express the _T. roseopersicina_ HoxYH hydrogenase in _S. elongatus_, the _hoxEFUYH_ gene cluster was cloned upstream of the _hupCDHIOF_ genes, resulting in plasmid pHoxhup-NSI (Fig. 3). pHoxhup-NSI with the combined gene cluster was transformed into _S. elongatus_ PW416 and Hyn5, to make strains Hoxhup and Hyn5/Hoxhup, respectively. _S. elongatus_ cultures were induced with IPTG, and protein extracts from lysed cells were used for immunoblots analysis. A 62 kDa protein band corresponding to the correct size of the mature form of HynL was observed (Fig. 4). In vitro hydrogen evolution assay was performed to determine if an active hydrogenase was produced. No activity was detected for Hoxhup or Hyn5/Hoxhup that contains additional _T. roseopersicina_ accessory genes _hynDhup/hupC1C2DEF_ (data not shown). Thus, maturation of a functional HoxYH from _T. roseopersicina_ requires additional accessory genes that are not able to be complemented by genes in the cyanobacterial host.

**Co-expression of _T. roseopersicina_ HynSL and AltDE accessory proteins in _S. elongatus_**

Since HynSL from AltDE was active when expressed in _S. elongatus_ RC41, we tried to co-express the _T. roseopersicina_ HynSL hydrogenase with accessory proteins from AltDE. Plasmid pWAV10 (Fig. 3) was constructed to carry only the 11 genes encoding AltDE accessory proteins and was introduced into the NSI locus of _S. elongatus_ strains Hyn4 and Hyn5 described above, creating Hyn4/Wav10 and Hyn5/Wav10, respectively. To serve as a control, pWAV10 was also introduced into the _S. elongatus_ hydrogenase knockout strain, PW416,
creating Wav10 that contained all AltDE accessory genes, but no structural genes. Expression of the HynL protein in these strains was verified by SDS-PAGE and Western blotting (Fig. 5A). A duplicate gel was stained with Coomassie blue to confirm equal loading in each lane as a loading control (Fig. 5B).

In vitro H₂ evolution assays were performed to determine whether the heterologously expressed T. roseopersicina HynSL hydrogenase was active when expressed in the cyanobacterium with the AltDE accessory proteins. Hydrogen evolution activity was detected in both Hyn4/Wav10 and Hyn5/Wav10 strains at similar levels in both strains (Fig. 5C). Activity from T. roseopersicina HynSL was approximately one tenth of the activity of AltDE HynSL (Figs. 2C and 5C). To determine if the AltDE accessory proteins were capable of producing an active T. roseopersicina HoxYH, the plasmid pWav10 was introduced into the S. elongatus strain Hoxhup. The resulting strain, Hoxhup/Wav10, did not produce any H₂ during in vitro hydrogen evolution assays after IPTG induction (Fig. 5C).

**Discussion**

We have expressed the [NiFe] hydrogenases from AltDE and T. roseopersicina in the heterologous host, S. elongatus PCC7942. [NiFe] hydrogenases have been previously expressed in heterologous bacterial hosts [14,15,25,26,27,28], but to our knowledge this is the first [NiFe] hydrogenase to be heterologously expressed in a cyanobacterium with a fully assembled active site. The ability to heterologously express properly assembled [NiFe] hydrogenases in cyanobacteria has been a technical barrier hindering widespread biotechnological application of hydrogenases with unique properties. In direct photobiological hydrogen production systems, electrons derived from water oxidation through oxygenic photosynthesis are used directly for hydrogen production without being stored as a fixed-carbon intermediate [29]. Development of such a system requires either low partial pressures of O₂ as has been achieved for brief periods with eukaryotic green algae [30], or the use of an O₂-tolerant hydrogenase. We have taken a step toward

---

**Figure 3. Diagram of constructs used in heterologous expression of T. roseopersicina HynSL in S. elongatus.** Arrows indicate the direction of open reading frames. The figure was drawn to scale. doi:10.1371/journal.pone.0020126.g003

**Figure 4. Detection of the T. roseopersicina hydrogenase large subunit HynL in S. elongatus.** Western blotting was performed on protein extracts from S. elongatus strains (wild-type PCC7942, Hyn4, Hyn5, Hyn5/Hup, and Hyn5/Hoxhup) by using anti-HynL antiserum. Hyn4/Hup was not included since the expression level in this strain was similar to that in Hyn4. Each lane contains 25 μg total proteins from cells treated with or without IPTG. Strain Hyn4 carries the T. roseopersicina genes hynSL, hynD, hupK, and hycC,C,DEF while strain Hyn5 carries the genes from Hyn4 as well as isp1 and isp2. Strain Hyn5/Hup contains the T. roseopersicina genes hupCDHlorf and the genes from Hyn5. Strain Hyn5/Hoxhup contains the T. roseopersicina genes hoxEFLUYH in addition to the genes from Hyn5/Hup. doi:10.1371/journal.pone.0020126.g004
The set of AltDE accessory proteins that was found to be required for maximal activity of AltDE HynSL when heterologously expressed in Escherichia coli included HypCABDDEF, the protease HynD, HupH, and a protein of unknown function, Orf2 [16]. The S. elongatus strain RC41 expressed the above set of accessory proteins along with Orf1 and Cyt. The AltDE proteins Orf1 and Cyt were not found to be necessary for maximal activity when HynSL was heterologously expressed in E. coli, but it is unknown whether these proteins affect the maturation efficiency of HynSL in S. elongatus PCC7942. The set of AltDE accessory proteins contained in Wav10 was the same as those found on RC41 and was also sufficient to allow for expression of a functional T. roseopersicina hydrogenase HynSL in S. elongatus. When expressed in S. elongatus, AltDE HynSL activity was higher than T. roseopersicina HynSL activity, and this may reflect lower levels of activity of the AltDE accessory proteins when acting on the T. roseopersicina HynSL. Similarly, lower levels of activity were detected when HynSL was expressed with the AltDE accessory proteins in E. coli compared to expression of AltDE HynSL [16].

We also attempted to express the bidirectional hydrogenase from T. roseopersicina, HoxYH, in S. elongatus. Even after co-expression with the entire set of known T. roseopersicina accessory proteins or with the accessory proteins from AltDE, no hydrogenase activity could be detected from strains expressing HoxYH. Missing from the accessory genes known to contribute to maturation of HynSL in E. coli [16] and was also identified in T. roseopersicina; however, the HupH sequences from AltDE and T. roseopersicina share only 11% similarity. It is unknown whether the two HupH proteins function similarly in their respective hosts. Absent from the group of known T. roseopersicina accessory genes is hypAB. These genes encode proteins that belong to the set of accessory proteins (HypABCDEF) that is found in all species containing [NiFe] hydrogenases [12]. HypAB function to add the Ni atom to the nascent [NiFe] catalytic site. In some systems, the absence of HypAB has been complemented by high concentrations of nickel, but these genes are required for maximal hydrogenase activity [32]. When the AltDE hypAB genes were co-expressed with in T. roseopersicina hynSL, hypD, hypC1C2DEF in E. coli, no hydrogenase activity was detected [16]. This suggests that the AltDE HypAB proteins alone cannot effectively interact with the rest of the T. roseopersicina accessory proteins and that the additional proteins (HupH or Orf2) encoded by Wav10 are important to the maturation of the T. roseopersicina hydrogenase.

We also attempted to express the bidirectional hydrogenase from T. roseopersicina, HoxYH, in S. elongatus. Even after co-expression with the entire set of known T. roseopersicina accessory proteins or with the accessory proteins from AltDE, no hydrogenase activity could be detected from strains expressing HoxYH. Missing from the accessory genes known to contribute to maturation of HoxYH is the endo-peptidase, HoxW. This peptidase activity is apparently not detected from strains expressing HoxYH. Although the HupH sequences from AltDE and T. roseopersicina share only 11% similarity. It is unknown whether these proteins affect the maturation efficiency of HynSL in S. elongatus PCC7942. The set of AltDE accessory proteins was not sufficient to allow for expression of a functional T. roseopersicina hydrogenase HynSL in S. elongatus. When expressed in S. elongatus, AltDE HynSL activity was higher than T. roseopersicina HynSL activity, and this may reflect lower levels of activity of the AltDE accessory proteins when acting on the T. roseopersicina HynSL. Similarly, lower levels of activity were detected when HynSL was expressed with the AltDE accessory proteins in E. coli compared to expression of AltDE HynSL [16].
Cells were grown under continuous illumination (40 µM m⁻² s⁻¹) at 28°C in 100 ml cultures with constant shaking or in 500 ml cultures with constant stirring and aeration. Cultures were induced with Isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 100 µM and NιC₂ at a final concentration of 0.3 µM for 24 hours before the cultures were used for experiments. Cultures of AltDE and *T. roseopersicina* were grown as previously described [11].

Plasmid construction and genetic manipulation of cyanobacteria

Plasmid pPW416 was constructed to knockout the hydrogenase structural genes *hoxIH* in *S. elongatus* PCC7942 [36]. This vector was designed to leave the upstream *hoxU* and downstream *hoxW* intact (Table 1). To make pPW416, a four piece ligation was performed using the following DNA pieces: 1) A PGR product containing resistance genes to erythromycin (EmR) and chloramphenicol (CmR) amplified from pRL2948a using primers EmCm-F and EmCm-R (Table 2) and digested with XhoI and SpeI, 2) A PCR product containing 1-kb of sequence upstream of *S. elongatus hoxF* amplified using primers Hox11 and Hox12 and digested with HindIII and Xhol, 3) a PCR product containing 1-kb of sequence downstream of *hoxH* amplified with

| Strain name | Features | Reference |
|-------------|----------|----------|
| DH10B | F- mcr Δ(mrr-hsdRMSC-mobC) Δ(nalR67)Δ(lacQΔlacI)Δ(lacY1ΔlacZΔM15) recA1 endA1 araD139 Δ ara leu7697 galU galK Δ-rysL ΔnuG | Invitrogen |
| Alteromonas macleodi “deep eutrophic” DSMZ 17117 | Wild type | [38] |
| Thiocapsa roseopersicina BB5 | Wild type | [39] |
| *Synechococcus elongatus* strains | | |
| *S. elongatus* PCC 7942 | Wild type | Pasteur Culture Collection |
| PW416 | PCC 7942, ΔhoxYH:EmR | This work |
| RC41 | AltDE hydrogenase operon inserted into NSI site [21] of strain PW416, EmR, SpR | This work |
| Hyn4 | hynShynUyhpsChypsCZhypsDhypsEhypsF from *T. roseopersicina* in NSI site [21] of strain PW416, EmR, KmR | This work |
| Hyn5 | hynShynUyhpsChypsCZhypsDhypsEhypsFisp2 from *T. roseopersicina* in NSI site [21] of strain PW416, KmR, EmR | This work |
| Hup | hupCDHorf from *T. roseopersicina* in NSI site of strain PW416, SpR, EmR | This work |
| HoxHup | hoxEFUYhupCDHorf from *T. roseopersicina* in NSI site of strain PW416, SpR, EmR | This work |
| Wav10 | orf1/cyt4/cyt3/hynD/hupU/hypC/hypA/hypB/hypD/hypF/hypE from AltDE in NSI site of strain PW416, SpR, KmR, EmR | This work |
| Hyn4/Wav10 | pWav10 construct in NSI site, pHyn4 construct in NSII site of strain PW416, SpR, KmR, EmR | This work |
| Hyn5/Wav10 | pWav10 construct in NSI site, pHyn5 construct in NSII site of strain PW416, SpR, KmR, EmR | This work |
| Hyn4/Hup | pHup construct in NSII site, pHyn4 construct in NSI site of strain PW416, SpR, KmR, EmR | This work |
| Hyn5/Hup | pHup construct in NSII site, pHyn5 construct in NSI site of strain PW416, SpR, KmR, EmR | This work |
| Wav10/HoxHup | pWav10 construct in NSI site, pHoxHup-NSII construct in NSII site of strain PW416, SpR, KmR, EmR | This work |

Plasmids:

- pRL2948a: mob, oriT, sacB, EmR, CmR
- pTRC-NSI: Cloning vector, SpR
- pTRC-NSII: Cloning vector, KmR
- pHoxHup-NSI: *T. roseopersicina hoxEFUYhupCDHorf cloned into pTRC-NSI*
- pHoxHup-NSII: *T. roseopersicina hoxEFUYhupCDHorf cloned into pTRC-NSII*
- pHox-NSI: *T. roseopersicina hoxEFUYhupCDHorf genes cloned into pTRC-NSI*
- PHyn4-NSII: *T. roseopersicina hynSl, hynD, hupK, hypC1, hypC2, hypDEF genes cloned into pTRC-NSII*
- PHyn5-NSII: *T. roseopersicina hynSl, hynD, hupK, hypC1, hypC2, hypDEF, isp1, isp2 genes cloned into pTRC-NSII*
- pWav10: orf1/cyt4/cyt3/hynD/hupU/hypC/hypA/hypB/hypD/hypF/hypE inserted into NSI site, ΔhynSl, cloned into pTRC-NSI, SpR
- pPW416: *S. elongatus hox region (ΔhoxYH:EmR) cloned in pUC19, EmR, CmR
- pRC41: orf1/cyt4/cyt3/hynD/hupU/hynShynC/hypA/hypB/hypD/hypF/hypE inserted into NSI site, cloned into pTRC-NSI, SpR

Table 1. Bacterial strains and plasmids used in this study.

Expression of [NiFe] Hydrogenases in Cyanobacteria

Bertani (LB) broth, or on LB agar plates supplemented with antibiotics as needed (spectinomycin, 50 µg ml⁻¹, kanamycin, 25 µg ml⁻¹, chloramphenicol, 25 µg ml⁻¹). Cyanobacteria were grown in BG11 liquid media [35] or on BG11 agar plates supplemented with antibiotics as needed (spectinomycin, 10 µg ml⁻¹, kanamycin, 10 µg ml⁻¹; erythromycin, 5 µg ml⁻¹; chloramphenicol (25 µg ml⁻¹). Cells were grown under continuous illumination (40 µM m⁻² s⁻¹) at 28°C in 100 ml cultures with constant shaking or in 500 ml cultures with constant stirring and aeration. Cultures were induced with Isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 100 µM and NιC₂ at a final concentration of 0.3 µM for 24 hours before the cultures were used for experiments. Cultures of AltDE and *T. roseopersicina* were grown as previously described [11].
primers Hox15 and Hox16 and digested with SpeI and XbaI, and 4) pUC19 digested with HindIII and XbaI. After transformation into E. coli, the resulting plasmid was subsequently digested with XbaI and ligated with an XbaI-digested RNA fragment from pRL448 containing a kanamycin resistance gene (KmR). The resulting plasmid, pPW416, was confirmed by restriction digest and DNA sequencing. The plasmid was then transformed into S. elongatus as previously described [31], and the resulting strain was called PW416. Double DNA recombination was verified by sensitivity to kanamycin, and the strain was segregated by streaking cells on progressively increasing concentrations of erythromycin. The absence of the hoxYH genes in the segregated PW416 strain was verified by PCR and southern hybridization.

The plasmid pHyn4 was previously described [16]. To make pHyn4-NSII, the entire cluster of T. roseopersicina genes in pHyn4 was digested with NdeI and AscI and ligated into a similarly digested pTRC-NSII. To make pHyn5-NSII, the isp1isp2 genes were amplified by PCR from T. roseopersicina genomic DNA using primers Isp-F and Isp-R, digested with SwaI and Ascl, and ligated into SwaI and Ascl-digested pHyn4-NSII. The resulting plasmid, pHyn5-NSII, was sequenced by restriction digest and DNA sequencing. The plasmid was then transformed into S. elongatus as previously described [31], and the resulting strain was called PW416. Double DNA recombination was verified by sensitivity to kanamycin, and the strain was segregated by streaking cells on progressively increasing concentrations of erythromycin. The absence of the hoxYH genes in the segregated PW416 strain was verified by PCR and southern hybridization.

To make pHyn4-NSII, the entire cluster of T. roseopersicina genes in pHyn4 was digested with NdeI and AscI and ligated into a similarly digested pTRC-NSII. To make pHyn5-NSII, the isp1isp2 genes were amplified by PCR from T. roseopersicina genomic DNA using primers Isp-F and Isp-R, digested with SwaI and Ascl, and ligated into SwaI and Ascl-digested pHyn4-NSII. The resulting plasmid, pHyn5-NSII, was sequenced by restriction digest and DNA sequencing. The plasmid was then transformed into S. elongatus as previously described [31], and the resulting strain was called PW416. Double DNA recombination was verified by sensitivity to kanamycin, and the strain was segregated by streaking cells on progressively increasing concentrations of erythromycin. The absence of the hoxYH genes in the segregated PW416 strain was verified by PCR and southern hybridization.

Hydrogenase activity assays

In vitro hydrogen evolution assays were performed as described in [28] with the following modifications. Cells (500 ml) were centrifuged, resuspended in 1 ml sonication buffer (10 mM Tris-HCl, pH 7, 0.5 mM EDTA, 1 mM DTT), and sonicated (under aerobic conditions) two times for 2 minutes each on ice and centrifuged to remove cell debris before being used for assays. Reactions were performed under anaerobic conditions at 30°C as described previously using the chemical electron donor, methyl viologen [28].

Protein techniques

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to [34]. Gels were either stained with Coomassie using the SimplyBlue SafeStain reagent (Invitrogen) or transferred to nitrocellulose for Western blotting using polyclonal rabbit antibodies specific for T. roseopersicina HynL and HynS as the primary antibodies [28,34].

Supporting Information

**Figure S1** Southern blot confirmation of the *S. elongatus* *hoxYH* mutant (PW416). After segregation on increasing antibiotic concentration, chromosomal DNA was digested with EcoRI and HindIII for Southern blotting from the following samples: Lane 1) Wild-type, 2) PW416-1, 3) PW416-2, and 4) PW416-3. A. Southern blot hybridized with a labeled PCR product amplified from hoxU. B. Southern blot hybridized with a labeled PCR product amplified from hox11. C. Restriction map of the wild-type *S. elongatus* hox11 region. D. Restriction map of the PW416 mutant hox11 region. (TIF)

**Figure S2** PCR confirmation of the *S. elongatus* *hoxYH* mutant (PW416). After segregation on increasing antibiotic concentration, chromosomal DNA was isolated and used for PCR. The templates used in each lane are the following: Lane 1) Wild-type, 2) PW416-1, 3) PW416-2, 4) PW416-3, 5) no template, 6) pPW416 plasmid DNA, and 7) *S. elongatus* PCC 7942 chromosomal DNA. A. PCR products amplifying hoxH using primers Hox23 and Hox24 (Table S1). B. Diagram of primer binding sites in *S. elongatus* C. PCR products amplifying hoxU through hoxW using primers Hox16 and Hox17 (Table S1). D. Diagram of primer binding sites in wild-type *S. elongatus* hox11 region. E. Diagram of primer binding sites in PW416 hox mutant. (TIF)

**Table S1** Primers used in the Supplemental Figures. (TIF)

**Table S2** *In vitro* hydrogen evolution activity assay on wild-type and segregated *hoxYH* mutant strains. (TIF)

Acknowledgments

We thank Dr. Yao Xu for kindly providing us with strains and expression vectors.

**Author Contributions**

Conceived and designed the experiments: PDW WAV YT JY P-CM HOS. Performed the experiments: PDW WAV YT JY P-CM HOS. Analyzed the data: PDW WAV YT JY P-CM HOS. Contributed reagents/materials/analysis tools: PDW WAV YT JY P-CM HOS. Wrote the paper: PDW QX.
