# Generation of a functional, semisynthetic [FeFe]-hydrogenase in a photosynthetic microorganism

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## Methods

### General

$[Fe_2(adt)(CN)_2(CO)_4]^{2-}$ (complex 1, adt = $-SCH_2NHCH_2S-$) was synthesized in accordance to literature protocols with minor modifications, and verified by FTIR spectroscopy[14]. All anaerobic work was performed in an MBRAUN Labmaster glovebox under argon atmosphere ([O2] ≤ 5 ppm).

### Strains and growth conditions

*Synechocystis* sp. strain PCC 6803 wild-type (*Synechocystis* WT) and *Synechocystis* sp. strain PCC 6803 *ΔhoxYH*[25] (*Synechocystis Δhox*) cells were routinely cultured in BG11 media at 30 °C, under a continuous irradiance of 40 µmol of photons $m^{-2}$ $s^{-1}$. *Synechocystis Δhox* CrHydA1 and *Synechocystis* WT CrHydA1 engineered cells were cultivated under similar conditions, except that the media was supplemented with kanamycin to a final concentration of 50 µg $mL^{-1}$. When required, after cultivation in BG11 media to O.D.750nm = 0.2 - 0.3, combined nitrogen step down was carried out by centrifuging cells at 5,000 × g for 5 mins, washing three times in $BG11_0$ media and suspending the cells to a final O.D.750nm = 0.2. The combined nitrogen starved cells were harvested 12 – 14 h after inoculation for further experiments.

All cloning was done using *Escherichia coli* strain DH5α Z1 grown at 37 °C in Luria broth (LB) liquid medium, and on plates containing LB medium solidified with 1 % (w/v) agar supplemented with 50 µg $mL^{-1}$ kanamycin.

**Plasmid construction and transformation**

All primers were supplied by Eurofins Genomics and are listed in **Supplementary Table 1**. The *hydA1*-teminator fragment was amplified using HydA-F1 and Hyd-R1 primers, template was kindly provided by Prof. Alfonso Jaramillo, University of Warwick, UK. The resulting fragment was amplified with HydA-F2 and Hyd-R2 primers and cloned into broad host range shuttle vector pPMQAK1[26] with restriction digestion (FastDigest EcoRI and SpeI, Thermo Fisher Scientific) and ligation (Quick Ligation Kit, New England Biolabs). The subsequent amplifications with forward primers HydA-F1 and HydA-F2 facilitated the two-step addition of a $Ptrc_{core}$[27] promoter and a bicistronic adaptor, BCD2[28], to the open reading frame of *hydA1*. The annotated sequence of the resulting *hydA1* expression unit can be seen in **Supplementary Note 1**. 1 μg of plasmid DNA was transformed into 100 μL, early mid log phase (~ O.D.750nm = 0.3) *Synechocystis Δhox* and *Synechocystis* WT cells. The cell suspensions were incubated at 30 °C in low light for 3-4 h after which they were spread on nitrocellulose filter placed on BG11 agar. After 24 h the filter was moved to BG11 agar supplemented with kanamycin (50 μg $mL^{-1}$). The resultant colonies were screened for positive clones by PCR and sequencing. Expression of pro-HydA1 was confirmed with Western Blot.

**Hydrogen production assays**

100 mL culture of O.D.750nm = 0.2 was centrifuged at 5000 rpm, concentrated 50 X by supplementing with fresh BGII or $BGII_0$ media containing appropriate concentrations of antibiotic, sparged with argon for 10 min and transferred to the glovebox. Where necessary, the media was supplemented with 30 μM DCMU and/or 100 μM DBMIB and/or 30 μM DCC and/or 5 mM glucose. Thereafter the cells were transferred into 8 mL glass vials, and 100 μg of complex **1** was added from a stock solution of **1** in potassium phosphate buffer (100 mM, pH 6.8). The volume of complex **1** solution was always maintained to less than 5 % (v/v) of the total reaction volume. Following 10 min incubation, the glass vials were sealed with rubber septa inside the glovebox and the samples were incubated at 30 °C on a rotary shaker either under 100 μmol photons $m^{-2}$ $s^{-1}$ or in darkness after wrapping the vials with aluminum foil. The evolution of hydrogen was monitored by injecting 100 μL of the headspace gas into a gas chromatograph.

**Hydrogen measurements by GC**

Hydrogen content was determined using a gas chromatograph (GC; PerkinElmer LLC, MA, USA) equipped with a thermal conductivity detector (TCD) and a stainless-steel column packed with Molecular Sieve (60/80 mesh). A calibration curve was established by injecting known amounts of hydrogen. The operational temperatures of the injection port, the oven and the detector were 100 °C, 80 °C and 100 °C, respectively. Argon was used as the carrier gas at a flow rate of 35 mL $min^{-1}$.


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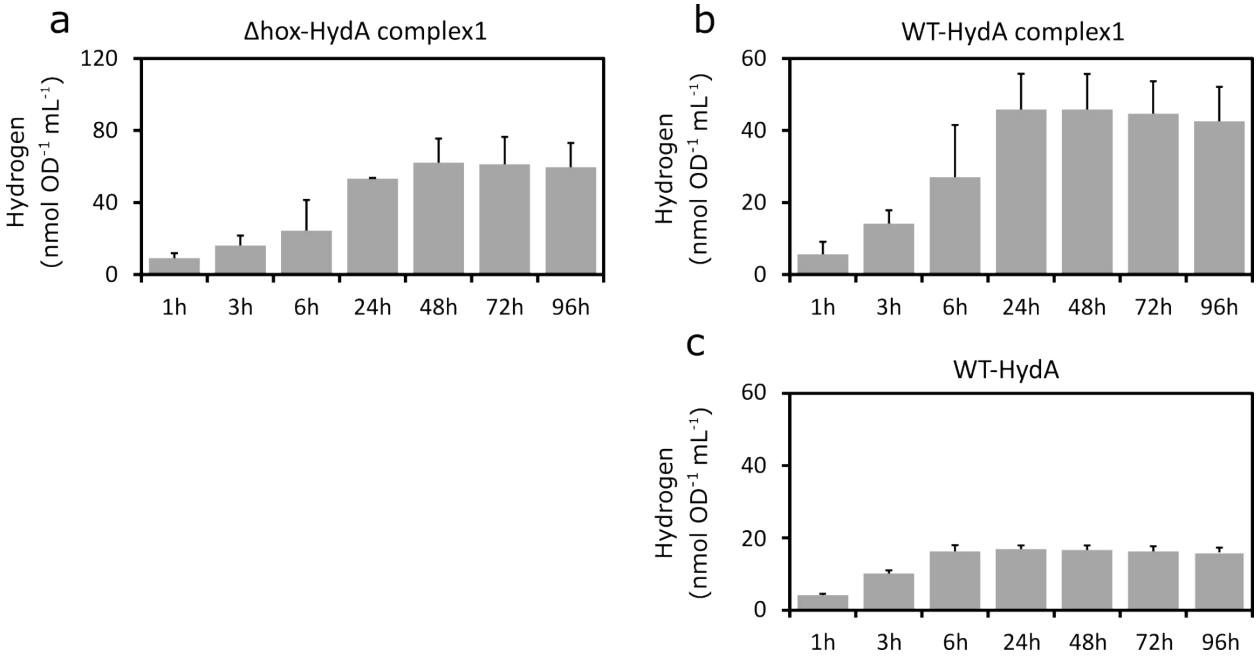
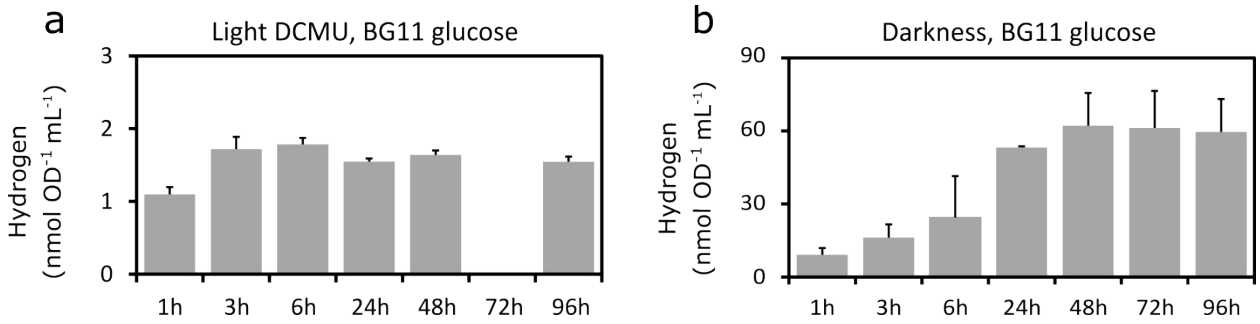
## Supplementary Information




**Supplementary Figure 1.** Time profiles of hydrogen accumulation from living cells of *Synechocystis* PCC 6803 WT and *Synechocystis* PCC 6803 *Δhox* expressing pro-HydA1 from *Chlamydomonas reinhardtii*. 100 µg (**a,b**) or 0 µg (**c**) **complex 1** was added to anaerobic cultures in media supplemented with glucose. Hydrogen was measured at indicated times. Columns represent accumulated hydrogen in nmol per mL culture, normalized to culture optical density (O.D.750nm). Data points represent means of 2-4 independent experiments. Error bars represent S.D.




**Supplementary Figure 2.** Time profiles of hydrogen accumulation from living cells of *Synechocystis* PCC 6803 *Δhox* harboring an activated CrHydA1, incubated in BG11 medium supplemented with glucose in (**a**) light with DCMU inhibition and (**b**) darkness. Columns represent accumulated hydrogen in nmol per mL culture, normalized to culture optical density (O.D.750nm). Data points represent means of 2-4 independent experiments. Error bars represent S.D.

**Supplementary Table 1**. Oligonucleotide primers

| Primer | Sequence 5'- 3' |
|---|---|
| HydA-F1 | agttcacttaaaaaggagatcaacaatgaaagcaattttcgtactgaaacatcttaatcatgctaaggaggttttctaatggctgctcctgctgctg |
| HydA-R1 | aaaaaaaaccccgccctgtcag |
| HydA-F2 | tttgaattcgagctgttgacaattgtgagcgctcacaatataatgtgtggaagggcccaagttcacttaaaaaggagatcaac |
| HydA-R2 | cgctactagtaaaaaaaaaccccgccctgtcag |

## Supplementary Note 1

*hydA1* expression unit sequence used in this study.

gagctgttgacaattgtgagcgctcacaatataatgtgtggaagggcccaagttcacttaaaaaggagatcaacaatgaaagcaattttcg
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tt

Legend:

P*trc*$_{core}$
Bicistronic design (BCD) including RBS I and RBS II
hydA1
Terminator (BB006)