

In vivo activation of an [FeFe] hydrogenase using synthetic cofactors

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Methods

Supplementary Figure 1.

In vivo hydrogen production observed following separation of supernatant from cells.

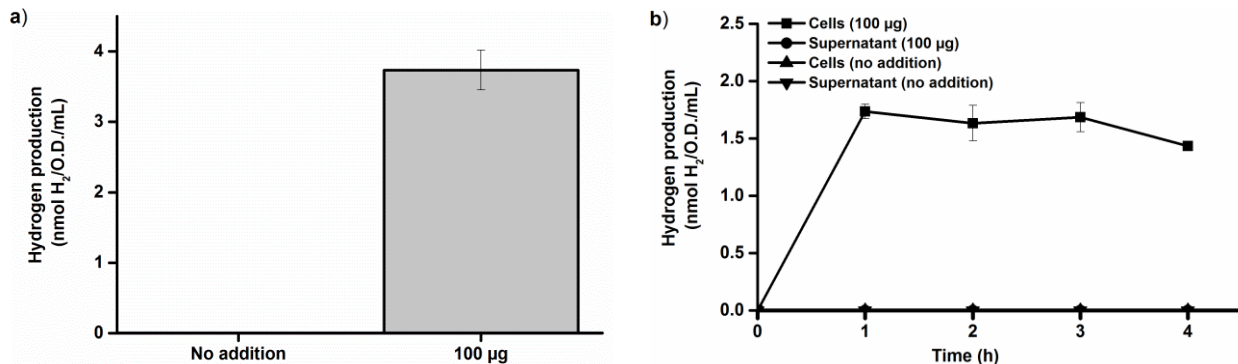


Figure S1. Cultures of *E. coli* BL21(DE3) cells containing the HydA1 vector were grown to O.D. \approx 0.2 at which time either 0 or 100 μ g (156 nmoles) of complex **1** was added. Hydrogen production was measured after 15 min to verify the successful activation of *C.r.* HydA1 (a). Thereafter, inside the glove box the cells were spun down, the supernatant filtered by 0.2 μ m syringe filters and collected in fresh glass vials. The cells were replenished with fresh media (LB supplemented with glucose). Both the cells and supernatant were incubated again and hydrogen production was measured every hour for the next 4 h. No formation of H₂ gas could be observed from the supernatant at any time point. In contrast renewed H₂ production was observed from the re-suspended activated cells (black squares) (b).

Supplementary Figure 2.

Comparison of hydrogen production observed after *in vivo* activation using the *E. coli* strains BL21(DE3) and FTD147, the latter lacking functional hydrogenases (Hyd-1, Hyd-2, Hyd-3).

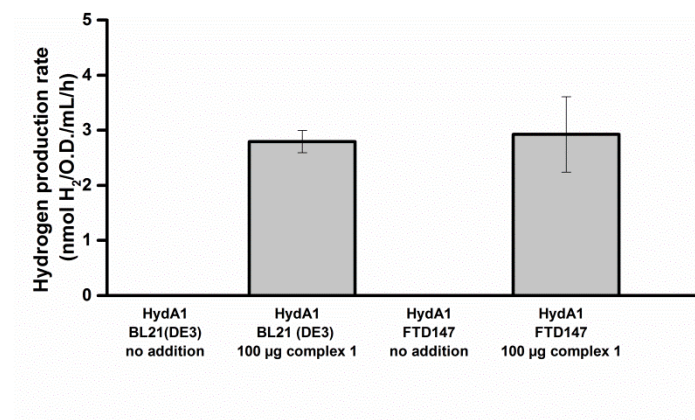


Figure S2. In a standard *in vivo* assay, either 0 or 100 µg (156 nmoles) of complex **1** was added to 100 mL cultures of BL21(DE3) or FTD147 cells containing the HydA1 vector. Rates calculated from the hydrogen produced at the 1 hour time point.

Supplementary Figure 3.

Controls of washing efficiency for *in vivo* to *in vitro* experiments involving 10 and 100 μg of complex **1**.

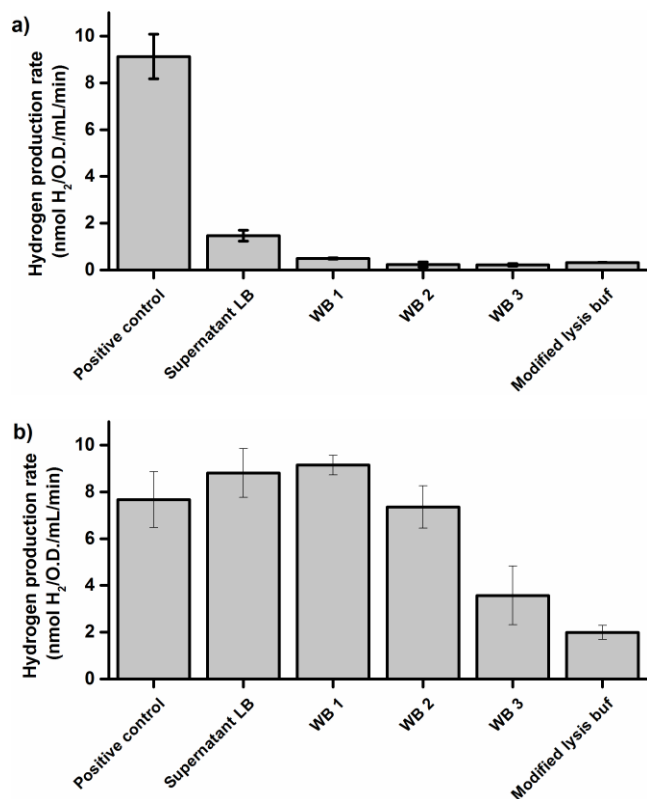


Figure S3. *In vitro* H₂ production rates observed following *in vivo* activation of fresh cells by the supernatant generated from the different wash steps used during the *in vivo* to *in vitro* assays from experiments using 10 μg (a) or 100 μg (b) of complex **1**. (Positive control: Fresh cells incubated with fresh LB + glucose + 10 or 100 μg of **1**; Supernatant LB: initial supernatant, containing LB, glucose and unreacted complex **1**; WB 1-3: the supernatant from wash steps 1-3 containing TBS wash buffer and unreacted complex **1**; Modified lysis buffer: Supernatant from the lysis buffer treatment, Triton X-100 and lysosome excluded to prevent cell lysis)

Supplementary Figure 4.

Additional controls of washing efficiency for *in vivo* to *in vitro* experiments involving 10 and 100 μg of complex 1.

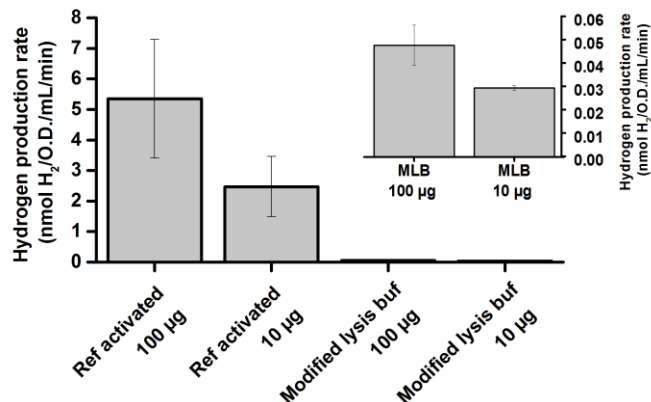


Figure S4. *In vitro* H₂ production rates observed following cell lysis of fresh cells in the presence of the modified lysis buffer obtained from *in vivo* to *in vitro* assays in experiments using 10 μg or 100 μg of complex 1 (rates multiplied by 4 to compensate for dilution). As reference, the rates observed in standard *in vivo* to *in vitro* assays using 10 μg or 100 μg of complex 1 are included (ref. activated 10 and 100 μg , also shown in Fig. 3 in main paper).

Inset: zoom in on rates observed for activation using the modified lysis buffer from experiments involving 10 μg or 100 μg of complex 1 (abbreviated MLB 10 or 100 μg).

Supplementary Figure 5.

Comparison of the HydA1 production in BL21(DE3) *E. coli* under non-induced (IPTG -) and under IPTG induced (IPTG +) conditions.

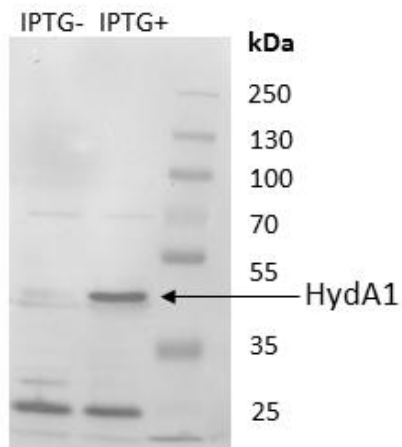


Figure S5. Western blot analysis to determine the difference in expression of the HydA1 protein under non-induced (IPTG -) and under IPTG induced (IPTG +) conditions, the HydA1 band appears at approx. 48 kDa (indicated by arrow). The increase in HydA1 expression appears to be significantly larger than the 2-3 fold increase observed in H₂ production. This discrepancy is tentatively attributed to incomplete formation of FeS clusters, resulting in a form of the enzyme unavailable for activation by complex **1**. For experimental details see the Methods section.

Supplementary Figure 6.

Hydrogen production observed following *in vitro* artificial activation of apo-HydA1 in IPTG induced *E. coli* BL21(DE3) cultures.

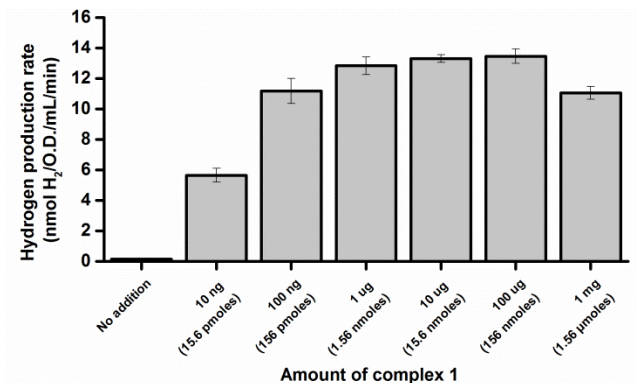
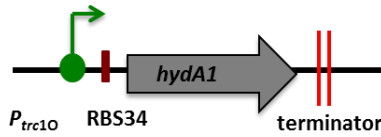


Figure S6. *In vitro* H₂ production rates observed following *in vitro* artificial activation of apo-HydA1 in crude cell extracts of IPTG induced *E. coli* cultures, rates calculated at the 45 min time point. Assay conditions: [MV] = 10 mM, [dithionite] = 20 mM, [kPi] = 60 mM, pH 6.8. All data points represent means of at least two independent repeats with duplicate samples from each culture, error bars \pm S.D.

Supplementary Figure 7.

Schematic overview of the *hydA1* gene construct.



The gene encoding the [FeFe] hydrogenase (*hydA1*) was expressed using the *P_{trc10}* promoter and biobrick part B0034 (RBS34). BB0015 was used as the transcriptional terminator.

Supplementary Note 1.

Sequence of the *hydA1* operon used in this study

hydA1 nucleotide sequence file

P_{trc10}-RBS34-*hydA1* stop codon-BB0015

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ttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataacaatttcacacataactagagaagaggagaaatactagatggc
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acctcgggtgggcctttctgcgttata
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** Non-highlighted regions indicate standard biobrick scars between individual parts

Supplementary Table 1.
Bacterial strains

Strain	Relevant genotype
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> B F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻m_B⁻)</i> λ(DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>] [<i>malB</i> ⁺] _{K-12} (λ ^S))
<i>E. coli</i> FTD147	<i>E. coli</i> K-12. F ⁻ , λ ⁻ , [<i>araD139</i>] _{B/r} , Δ(<i>argF-lac</i>)U169, <i>e14-</i> , <i>flhD5301</i> , Δ(<i>fruK-yeiR</i>) 725(<i>fruA25</i>), <i>relA1</i> , <i>rpsL150</i> (Str ^R), <i>rbsR22</i> , Δ(<i>fimB-fimE</i>)632(: <i>IS1</i>), <i>deoC1</i> , Δ <i>hyaB</i> , Δ <i>hybC</i> , Δ <i>hycE</i>

Supplementary methods

General

$[\text{Fe}_2(\text{CN})_2(\text{CO})_4(\text{adt})]^{2-}$ (adt = $^-\text{SCH}_2\text{NHCH}_2\text{S}^-$) (1) and $[\text{Fe}_2(\text{CN})(\text{CO})_5(\text{adt})]^-$ (adt = $^-\text{SCH}_2\text{NHCH}_2\text{S}^-$) (2) were synthesized in accordance to literature protocols with minor modifications, and verified by FTIR spectroscopy.¹⁻³ All anaerobic work was performed in an MBRAUN Labmaster glovebox under argon atmosphere ($[\text{O}_2] \leq 5$ ppm).

Bacterial growth conditions

Seed inoculum was cultivated overnight at 37 °C with shaking in Luria–Bertani (LB) medium. Kanamycin (50 µg/mL) was added to the LB medium for the selection and propagation of the HydA1-pPMQAK1 plasmids.

Construction of plasmids and strain

The [FeFe] hydrogenase gene (*hydA1*) from *Chlamydomonas reinhardtii* codon optimized and synthesized for expression in *E. coli* and *Synechocystis* PCC 6803, was kindly provided by Prof. Alfonso Jaramillo, University of Warwick, UK (see additional information, **Supplementary Fig. 7** and **Supplementary Note 1** for construct overview and nucleotide sequences). Cloning of the *C. r. hydA1* into pPMQAKI BioBrick vector under the effect of the P_{trc10} promoter was carried out using BioBrick standard assembly (Phillips I., Silver P., A New Biobrick Assembly Strategy Designed for Facile Protein Engineering. Dspace 2006, <http://hdl.handle.net/1721.1/32535>). Two *E. coli* strains were transformed with the *C. r. hydA1* plasmid, BL21(DE3) and FTD147⁴ (see additional information, **Supplementary table 1**), the latter strain was kindly provided by Prof. Frank Sargent at the Division of Molecular and Environmental Microbiology, College of Life Sciences, University of Dundee, Dundee, UK. An empty vector (EV) control was also developed

by transforming *E. coli* BL21 (DE3) with a pPMQAK1 vector where the lethal gene, *ccdB* was removed by digestion with *EcoRI* and *PstI*.

Hydrogen production assays

Seed cultures of experimental cells were cultivated aerobically, overnight in LB medium supplemented with antibiotics. The following day, the cultures were diluted 200 x and grown aerobically in fresh LB medium to an early log phase (O.D. ~ 0.2) at 37 °C. Where necessary 0.5 mM IPTG was added to the growth media at the time of inoculation.

a) In vivo measurements

100 mL LB culture per sample were centrifuged at 1575 g and concentrated 200 x, sparged with argon for 10 min and transferred to the glovebox. The cells were transferred into 8 mL glass vials, and then the appropriate amount of complex **1** or **2** was added from stock solutions of the complexes, **1** in potassium phosphate buffer (100 mM, pH 6.8) and **2** in a 1:1 mix of potassium phosphate buffer and DMSO. The volume of complex solution was always maintained to less than 5 % of the total reaction volume. Following a 10 min incubation, the reaction volume was made up to 2 mL with LB enriched with 20 mM glucose. The glass vials were sealed with rubber septa inside the glovebox and the samples were incubated at 37 °C on a rotary shaker (220 rpm). The evolution of hydrogen was monitored every hour by injecting 100 µL of the headspace gas into a gas chromatograph. In order to ensure that H₂ production originated from intracellular processes and not from enzymes released into solution, control experiments were performed where activated cells treated with complex **1** were separated from the supernatant via a combination of centrifugation and filtration. H₂ generation was clearly observable from the cell fraction after it was replenished with fresh media (LB supplemented with glucose), while no formation of H₂ gas could be observed from the supernatant at any time point (Figure S1).

b) In vitro measurements

The assay was performed following a slightly modified literature protocol.⁵ 100 mL cultures were harvested upon reaching O.D. ~ 0.2, sparged with argon for 10 min and treated with 300 µL of anaerobic lysis buffer (60 mM Tris buffer, pH 7.8; 0.6 mg / mL lysozyme, and 0.2 % Triton X-100) inside the glovebox. The cells were then transferred to 2 mL gas tight vials, sealed and lysed by 3 freeze-thaw cycles in an ethanol/dry ice bath. The cell lysate was transferred into 8 mL gas tight glass vials and re-suspended in 1.42 mL assay buffer (84.5 mM potassium phosphate buffer, pH 6.8, 1.4 % Triton X-100 and 14 mM methyl viologen, for final concentrations see figure legends). The appropriate amount of complex **1** was added from a stock solution as described above, the samples were then sealed and incubated at room temperature for 10 min. Thereafter the reaction was initiated by adding 200 µL of 0.2 M sodium dithionite. The reactions were incubated at 37 °C using a water bath. The evolution of hydrogen was first recorded after 15 min by injecting 100 µL of the headspace into a gas chromatograph followed by subsequent samples every 30 min.

c) In vivo to in vitro measurements

100 mL cell cultures were cultivated and treated as described in the protocol for *in vivo* hydrogen measurements. Following the addition of complex **1** to the cell cultures, the samples were incubated at 37 °C in a shaker for 1 h. The evolution of hydrogen was monitored by injecting 100 µL of the headspace into the gas chromatograph and thereafter the experimental vials were transferred into the glovebox for *in vitro* treatment.

The cells were spun down anaerobically and washed three times with 1 mL wash buffer (100 mM Tris, pH 7.5 and 150 mM NaCl). Following this treatment, the lysis and *in vitro*

measurements were conducted analogously to the MV/dithionite method described earlier with the exception that no further complex was added to the reactions. The reactions were incubated at 37 °C using a water bath. The evolution of hydrogen was first recorded after 15 min by injecting 100 µL of the headspace into a gas chromatograph followed by subsequent samples every 30 min.

d) In vivo to in vitro measurements – washing controls

In order to verify the quality of the washing protocol two separate control experiments were performed, shown in figures S3 and S4. In both cases a standard *in vivo* to *in vitro* experiment as described earlier in section “*c) In vivo to in vitro measurements*” was conducted by adding the desired amount of complex **1** (10 or 100 µg). After activation of the HydA1 proteins with complex **1** for 1 h at 37 °C, *in vivo* hydrogen production was measured. After this step the LB media, containing residual complex **1** was separated from the cells by centrifugation for 2 min at 2408 g in the glove box (denoted supernatant LB in Fig. S3). Subsequently, the cell pellet was washed three times with 1 mL wash buffer (100 mM Tris pH 7.5 and 150 mM NaCl) (denoted WB 1-3 in Fig. S3) and once with 1 mL modified lysis buffer consisting of 60 mM Tris pH 7.8 and 150 mM NaCl (denoted modified lysis buffer in Figs S3 and S4). The modified lysis buffer is introduced into the protocol to mimic the solution into which the hydrogenase enzyme is released upon lysis, and is expected to contain any residual complex **1** still being released from the cell after the three wash steps. In the modified lysis buffer, in order to prevent actual cell lysis, which would complicate the control due to release of HydA1 from the cells, Triton X-100 and lysozyme were omitted and instead replaced with 150 mM NaCl to maintain cell osmolality. After each wash and centrifugation step, the supernatants were collected and stored anaerobically at room temperature. In the first set of control experiments the collected supernatant fractions

were then added individually to different vials containing 100 mL cells (O.D. = 0.20 ± 0.02) concentrated 200 x. The cells were activated with the different wash fractions at room temperature for 15 min. As a positive control, a set of fresh cells were incubated with 1 mL fresh LB supplemented with 20 mM glucose and the corresponding amount of complex **1** (denoted positive control in Fig. S3). After activation of the cells with the washing fractions, the supernatant was separated from the cells with 2 min 2408 g centrifugation. The cells were lysed and *in vitro* measurements were conducted similar to the *in vitro* method described earlier with the exception that no further complex **1** was added to the reactions. The reactions were incubated at 37 °C in a water bath. The evolution of hydrogen was first recorded after 15 min by injecting 100 µL of the headspace into a gas chromatograph followed by subsequent samples every 30 min.

In the second set of control experiments fresh cells were lysed in the presence of the modified lysis buffer obtained from an *in vivo* experiment as described above (using complex **1**, 10 or 100 µg). After the last wash with modified lysis buffer, the cells were centrifuged and the supernatant was collected and added to a new 100 mL batch of fresh cells (O.D. = 0.20 ± 0.02) concentrated 200 x. The reaction was incubated for 15 min following which ¼ of this reaction volume (cells + supernatant) was transferred to a gas tight screw cap tube and 300 µL of lysis buffer was added. Following this, the routine *in vitro* activity analysis was conducted. The reactions were incubated at 37 °C in a water bath. The evolution of hydrogen was first recorded after 15 min by injecting 100 µL of the headspace into a gas chromatograph followed by subsequent samples every 30 min.

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. Nitrogen was used as the carrier gas at a flow rate of 35 mL min⁻¹.

SDS-PAGE/Western immunoblot to compare the protein content between the non-induced and induced cells

BL21(DE3) cells containing the HydA1-pPMQAK1 plasmid were grown in LB medium until O.D. = 0.2 at 37°C under aerobic conditions in the presence or absence of IPTG. The IPTG treated samples were supplemented with 0.5 mM IPTG at the time of inoculation. The protein content was evaluated by 10% SDS-PAGE in Laemmli's buffer system in SE Mighty Small II unit (Hofer) using Page Ruler Plus Prestained Protein Ladder (Thermo Scientific) as standard. The proteins were blotted onto PVDF membrane at 130 mA for 2h using a TE Mini Tank Transfer Unit (GE Healthcare). The blotted membrane was overnight treated with rabbit-anti-HydA1 IgG (Agrisera AS09 514) at 1 : 5000 dilution, and for 1 hour with goat-anti-rabbit IgG-HRP Conjugate (Bio-Rad) at 1 : 10000 dilution. The interactions were detected with Immun-Star-HRP Chemiluminescent kit (Bio-Rad). Chemiluminescence was measured in a ChemiDoc XRS system (Bio-Rad) using exposure times between 10 s and 5 min.

Notes and references

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