Hydrogen overproducing nitrogenases obtained by random mutagenesis and high-throughput screening

Emma Barahona, Emilio Jiménez-Vicente & Luis M. Rubio

When produced biologically, especially by photosynthetic organisms, hydrogen gas (H$_2$) is arguably the cleanest fuel available. An important limitation to the discovery or synthesis of better H$_2$-producing enzymes is the absence of methods for the high-throughput screening of H$_2$ production in biological systems. Here, we re-engineered the natural H$_2$ sensing system of *Rhodobacter capsulatus* to direct the emission of LacZ-dependent fluorescence in response to nitrogenase-produced H$_2$. A lacZ gene was placed under the control of the hupA H$_2$-inducible promoter in a strain lacking the uptake hydrogenase and the *nifH* nitrogenase gene. This system was then used in combination with fluorescence-activated cell sorting flow cytometry to screen large libraries of nitrogenase Fe protein variants generated by random mutagenesis. Exact correlation between fluorescence emission and H$_2$ production levels was found for all automatically selected strains. One of the selected H$_2$-overproducing Fe protein variants lacked 40% of the wild-type amino acid sequence, a surprising finding for a protein that is highly conserved in nature. We propose that this method has great potential to improve microbial H$_2$ production by allowing powerful approaches such as the directed evolution of nitrogenases and hydrogenases.

Biological H$_2$ production is a promising source of renewable energy. Microorganisms produce H$_2$ by the activity of nitrogenases and hydrogenases. Nitrogenases catalyze the reduction of N$_2$ with the limiting stoichiometry N$_2$ + 8H$^+$ + 8e$^-$ + 16MgATP + 16H$_2$O $\rightarrow$ H$_2$ + 2NH$_3$ + 16MgADP + 16Pi in a process known as biological nitrogen fixation, which produces H$_2$ as a by-product. On the other hand, hydrogenases catalyze the reversible 2 H$^+$ + 2e$^-$ $\leftrightarrow$ H$_2$ reaction. H$_2$ metabolism has been well studied in purple non-sulfur bacteria (PNS), a group of microorganisms notable for their metabolic versatility. PNS can grow photoautotrophically, photoheterotrophically, chemoorganotrophically, and chemolithotrophically with H$_2$ as an electron donor and O$_2$ as an electron acceptor. H$_2$ production by PNS generally occurs during photoheterotrophic anaerobic growth and is mainly due to nitrogenase.

In *R. capsulatus*, a two-component signal transduction system activates the transcription of the *hup* gene cluster in the presence of H$_2$. The H$_2$-sensing system comprises three elements: a cytosolic [Ni-Fe] hydrogenase, HupUV; a histidine kinase, HupT; and a response regulator, HupR. In the absence of H$_2$, HupUV and HupT...
interact, causing HupT autophosphorylation and the transfer of a phosphate group to HupR, which in this state is unable to activate the transcription. In the presence of H₂, HupUV binds H₁ and HupT is released. In this state, phosphotransfer between HupT and HupR is not favored, and the unphosphorylated HupR binds to promoter DNA and activates the transcription of uptake hydrogenase genes. Similar regulatory systems are found in other bacterial species such as *Bradyrhizobium japonicum* and *Ralstonia eutropha*.

Due to its great commercial significance, nitrogenase has been the subject of extensive biochemical, genetic and structural analyses. Nonetheless, it has proven difficult to find or engineer strains of microbes carrying nitrogenases with significantly increased H₂ production efficiency. In this work, we present a method for the high-throughput selection of nitrogenase variants with enhanced H₂ production. An *R. capsulatus* strain has been re-engineered to generate a fluorescent signal in response to nitrogenase-produced H₂. A combination of *nifH* random mutagenesis and fluorescence-activated cell sorting (FACS) is then used to select the H₂-overproducing nitrogenase variants in the *R. capsulatus* sensor strain (Fig. 1A).

**Results**

**Genetic modules for the selection of H₂-overproducing nitrogenase variants.** The construction of two genetic modules was required to perform high-throughput experiments to obtain nitrogenase variants with improved H₂ production: a module expressing over a million random variants of dinitrogenase reductase (NifH) per experiment and a reporter module directing the emission of a visible signal in response to H₂.

The reporter module was constructed in four steps. In a first step, an 874-bp DNA fragment comprising a promoter sequence upstream of *hupA* was translationally fused to *lacZ* in the replicative vector pMP220 to generate pRH802. As illustrated in Fig. 1A, the expression from *hupA* promoter is activated by HupR in response to H₂. The *in vivo* β-galactosidase activity of *R. capsulatus* cells harboring pRH802 (RC4) was 600-fold higher than that of the control strain RC3 (carrying pMP220) and responded positively to the presence of 10% H₂ in the culture gas phase, which confirmed the induction of the transcription from *hupA* by H₂ (Fig. S2A).

In a second step, the reporter dose was adjusted by integrating *hupA:*lacZ between *nifH* and *hupA* in the *R. capsulatus* chromosome to generate the S1 strain (Fig. S1). The S1 strain exhibited a much lower β-galactosidase activity background level and a larger fold increase in the activity in response to the external H₂ than RC4 (Fig. S2B).

In a third step, the reporter response to H₂ was adjusted by mutating the genes involved in the H₂ signal transduction pathway and metabolism. S1 derivative strains lacking *hupAB* structural genes for the uptake-hydrogenase (RC25-S1, also termed S2), the H₂ response regulator encoding gene *hupR* (RC54-S1), or the histidine kinase gene *hupT* (RC24-S1) were generated, and their responses to H₂ were analyzed (Fig. 1B and Fig. S3), obtaining the following results. First, the response to 10% exogenous H₂ improved 5-fold in the Δ*hupAB* strain S2 compared to in S1; second, the constitutive activation of *PhupA* was observed in the Δ*hupT* strain RC24-S1; and third, no response to H₂ was observed in the Δ*hupR* strain RC54-S1. These results were in agreement with previous reports indicating proper control by the *R. capsulatus* H₂-sensing system and therefore permitting further H₂ sensor development through the use of strain S2.

The deletion of *hupAB* genes in the RC25 strain completely eliminated its *in vivo* uptake hydrogenase activity (nearly compared to 2504 ± 450 nmol H₂ h⁻¹ OD₆00⁻¹ in the wild-type strain). Thus, in contrast to the wild-type strain, the S2 sensor strain (derived from RC25) evolved high levels of H₂ under diazotrophic growth conditions (Fig. S2C) in spite of having similar levels of *in vivo* nitrogenase activity (Fig. S2D). In addition, the X-gal and H₂-dependent signal-to-noise ratio was much better in S2 than in S1 cultures (Fig. S2E). It was therefore concluded that the elimination of the uptake hydrogenase activity would facilitate the detection of intracellular H₂ produced during the nitrogen fixation process.

The S2 response to the H₂ produced by the nitrogenase was evaluated in intact bacterial cells by using a fluorescent MUG-dependent β-galactosidase activity assay, which permitted high-throughput growth and screening in a 96-well format as well as the recovery of viable selected clones. *R. capsulatus* strains were grown in 96-well microplates both under non-diazotrophic and diazotrophic conditions inside a glovebox, and their β-galactosidase activities were determined. Figure 1C shows that the β-galactosidase activity in S2 was 15-fold higher under diazotrophic conditions compared to non-diazotrophic conditions, validating its use as a biosensor. Importantly, all tested strains with mutations in H₂, the metabolism or signal transduction pathway responded identically to nitrogenase-produced H₂ and exogenous added H₂ (compare Fig. 1C to Fig. S3).

Finally, the removal of endogenous *nifH* was necessary to use the sensor in combination with the genetic module consisting of an expression library of *nifH* variants (Fig. S4). The S3 sensor strain (Δ*nifH* Δ*hupAB* PhupA::lacZ) β-galactosidase activity levels in response to H₂ were identical to those of S2 (Fig. 1B), demonstrating that the absence of *nifH* did not modify the capacity to detect H₂. Importantly, the introduction of the pRH576 expression vector carrying a wild-type copy of *nifH* under the control of its own promoter restored the *in vivo* nitrogenase activity of strain S3 (Fig. S5), validating its use to screen the activity of the *nifH* variants in S3. Interestingly, a general decrease in the nitrogenase activities was observed in the sensor S3-derived strains with respect to the wild-type *R. capsulatus*. This effect was associated with carrying the expression vector pBBR1MCS-3 (Fig. S6).

The second genetic module consisted of an expression library of randomly generated nitrogenase variants. The mutation analysis was initially constrained to *nifH* because the limiting steps in the nitrogenase catalysis are the dissociation of the NiFeH and NiFeDK components and the release of Pi from NiFeH₂. Random *nifH* variants (*nifHv*) having an average of 5 amino acid changes (Table S1) were generated by error-prone PCR, ligated into pRH602, and introduced into *E. coli* DH15α to obtain *PnifHv::nifH* expression libraries (~ 4 × 10⁶ clones per library). No obvious mutational hotspots were observed after sequencing 20 *nifHv* variants per library.
Finally, the two genetic modules were combined by introducing the \( nifH \)-expression libraries into \( R. \) \( capsulatus \) sensor strain S3. The mating process resulted in \( \sim 8 \times 10^5 \) clones per library, a number that required high-throughput screening methods.

**High-throughput selection of \( H_2 \)-overproducing \( nifH \) variants.** \( R. \) \( capsulatus \) S3 libraries expressing \( nifH \) variants (\( nifHv-S3 \)) were grown under nitrogenase-derepressing conditions inside a glovebox. After treatment with fluorescein di-\( \beta \)-D-galactopyranoside (FDG), culture samples were screened by FACS flow cytometry. Approximately \( 2 \times 10^5 \) events were processed per sample. \( R. \) \( capsulatus \) cells emitting fluorescence at levels significantly higher than the main population (0.024% of total population, see P2 area in Fig. 2A) were sorted by the cytometer into separate wells of 96-well microplates containing growth medium.
S3 derivatives transformed with empty expression vector (pRHB602-S3) or wild-type nifH-containing expression vector (pRHB576-S3) were used as negative and positive controls, respectively. pRHB602-S3 populations emitted the lowest average level of fluorescence, and only 0.008% of the population was found above the set threshold. On the other hand, the average fluorescence emission in the pRHB576-S3 populations was the highest, although only 0.0039% of the population was above the selective threshold.

The nifHv-S3 cells sorted by FACS were then cultured under nitrogenase-derepressing conditions using serine as a poor nitrogen source inside a glovebox for a secondary MUG-based fluorescence screen. Growth was observed in approximately 25% of the inoculated wells. Figure 2B shows the β-galactosidase activity levels of 67 nifHv-S3 cultures along with those of the pRHB602-S3 (blue) and pRHB576-S3 (red) cultures used as controls. The β-galactosidase activity was 2 to 12-fold higher in the nifHv-S3 cultures than in the pRHB576-S3 strain (carrying wild-type nifHv), Importantly, 85% of the nifHv-S3 clones harbored an expression vector with a nifHv gene, as determined by PCR analysis. The strong correlation of the results from the FACS primary screening and the secondary MUG screening in the 96-well format validates the use of flow cytometry as a high-throughput method to screen nifHv-S3 libraries.

Nitrogenase-dependent H2 overproduction in selected strains with nifH variants. Based on the β-galactosidase activity levels, R. capsulatus strains V1, V7, V8, V10, V17, V18, V20 and V21 (Fig. 2B, black bars), carrying the corresponding nifH variants, were selected for in vivo H2 production assays. All these strains produced more H2 than the pRHB576-S3 strain carrying wild-type nifH (Fig. 3A), with the V1 and V7 strains consistently producing up to 10-fold more H2. Importantly, the acetylene reduction activity was almost abolished in the strains carrying H2-overproducing nifH variants (Fig. 3B), a result consistent with the random mutagenesis process designed to select only for improved H2 production. The ratio of H2 to ethylene production in V7 strain was 6000-fold higher than in pRHB576-S3. Neither H2 nor ethylene was produced in the absence of nifH (see strain pRHB602-S3 in Fig. 3), indicating that the H2 production was dependent on the nifH expression.
Figure 3. Characterization of selected nifHv-S3 strains. H$_2$ production (A) and acetylene reduction activity (B) of highly fluorescent R. capsulatus strains carrying nifH variants. Strains pRHB602-S3 (no nifH) and pRHB576-S3 (wild-type nifH) were used as controls. Data represent the mean ± SD (n = 4). (C) Stereoview overlap of 3D-structural models for R. capsulatus NifH (purple) and the NifH-V7 variant (green). Both proteins are expected to form homodimers with a [4Fe-4S] cluster at the subunit interface. (D) Correlation of H$_2$ production (nmol ml$^{-1}$ hour$^{-1}$) and presence of NifH-V7. Data represent the mean ± SD (n = 4). (E) Immunodetection of nitrogenase components NifH and NifDK in strains carrying or lacking the NifH-V7 variant. pRHB602-S3 and pRHB576-S3 were used as controls. Lower panel shows protein loading in each sample as determined by the Coomassie staining of the corresponding SDS gels.
The nature of the mutations in the nifH-V1 and nifH-V7 variants was determined by DNA sequencing (Table S2 and Fig. S7). A frame shift mutation was found in codon 172 of nifH-V7 that would result in a NifH protein lacking 124 amino acids at its C-terminus. In addition, NifH-V7 would carry G32C and A107T amino acid substitutions. This was surprising because NifH is a highly conserved protein, and such a drastic truncation would be expected to yield an inactive protein. An overlap of the three-dimensional models of R. capsulatus NifH and NifH-V7 is shown in Fig. 3C. The overall protein overlap (4–171 amino acid residues) and an RMSD value of 0.900 Ångstroms for the protein backbone atoms indicate very similar model architectures. However, subtle differences were observed in the loops near the [4Fe-4S] cluster, where residues K43, A44, A100, G101, R102 and G115 did not overlap.

To confirm that the H₂ overproduction in V7 was dependent on the activity of both Mo-nitrogenase component proteins, the following two experiments were performed. First, the nifH-V7 expression plasmid was cured from the V7 strain by passing five times under a nonselective media. Plasmid elimination was confirmed by PCR analysis and by growth inhibition on media supplemented with tetracycline (Tc). The resulting strain, V7C, was unable to produce H₂ above the levels of the pRH602-S3 control strain (0.42 nmol H₂ h⁻¹ OD₆₀₀⁻¹ ml⁻¹) (Fig. 3D). The reintroduction of the nifH-V7 expression plasmid by mating completely restored the H₂-producing activity (compare H₂ productions of V7 and V7C in Fig. 3D). The presence of a truncated form of NifH in V7 and its elimination in V7C were confirmed by an immunoblot analysis (Fig. 3E). This analysis also showed that all strains accumulated similar levels of nitrogenase component proteins, ruling out the possibility of increased accumulation underlying the H₂ overproduction by V7.

Second, R. capsulatus sensor strains lacking either nifH (strain S3) or nifHDK (strain S5) were complemented with an expression plasmid harboring nifH-V7. While the S3-V7 cultures produced large amounts of H₂ background levels were detected in the S5-V7 cultures (Fig. S8). This result demonstrates that the NifH-V7 dependent H₂ production also requires the presence of NifDK and is not due to the activity of other cellular proteins, such as the Fe-only nitrogenase.

Discussion

There are many reports of metabolic engineering aiming to increase microbial H₂ production. Genetic approaches include deleting hydrogenase and/or nitrogenase structural and regulatory genes20–25, eliminating Rubisco26, lowering intracellular O₂ levels27, re-engineering hydrogenase to increase its tolerance to O₂28, and modifying nitrogenase substrate selectivity by site-directed mutagenesis29,30. Nonetheless, it has been difficult to find or engineer vastly improved H₂-overproducing enzymes or microbial strains.

Directed evolution mimics biological evolution in the laboratory and is an effective approach to change enzyme properties such as catalytic turnover31. Directed evolution requires a strategy to generate libraries with a large number of variants and a method capable of screening or selecting for the best variants in a large pool. In this work, we have combined the random mutagenesis of nifH and high-throughput screening to improve nitrogenase H₂-producing activity. We hypothesized that the nitrogenase H₂ production could be greatly improved if no selection was applied to maintain the N₂-reducing activity. The nifH gene was selected to obtain a proof of concept because the limiting steps in nitrogenase catalysis are the NifH/NifDK complex dissociation and the release of Pi from NifH. FACS flow cytometry was used as a high-throughput method to select H₂-overproducing NifH variants from the pool. A genetic module endowing a NifH-expressing sensor strain with the capacity to emit light in proportion to the amount of H₂ detected was required for this selection. The screening of bacterial libraries by FACS flow cytometry had been performed before in directed evolution procedures32.

A number of H₂-overproducing NifH variants were obtained. Perhaps the most interesting was the V7 variant, which lacks 124 amino acid residues at the C-terminus of the protein (Fig. 3C). In wild-type NifH, ATP binding and hydrolysis is required for electron transfer to NifDK during catalysis2. Interestingly, NifH-V7 lacks all residues shown to enable hydrogen bonding to the adenine base (Asp185, Gln218, and Gln236 in the other biological and chemical methods have also been developed to detect H₂-producing microorganisms. Chemochromic transparent sensor films that turn blue in the presence of H₂ and indicators consisting of a colo-

Conclusions

In this work, we have re-engineered the natural H₂-sensing system of R. capsulatus and combined it with FACS for the high-throughput selection of nitrogenase variants with enhanced H₂ production that independently retain their N₂ fixation activity. This method allows screening 10⁵–10⁶ variants per experiment, thus permitting enzyme improvement by directed evolution. This technology possesses great potential to identify nitrogenase amino acid substitutions leading to H₂-overproducing variants that could be mimicked in nitrogenases from other microorganisms, expanding the impact of the findings. In addition, it might be used for the genome-wide screening of mutations leading to enhanced H₂ production in R. capsulatus.

Materials and Methods

β-galactosidase activity assays. Transcription from PhupA was estimated by measuring β-galactosidase activity of R. capsulatus strains carrying PhupA::lacZ transcriptional fusions. Cells were cultured at 30 °C for 7 h,
transferred to assay tubes (0.7 ml of culture), permeabilized by addition of 20 μl of chloroform and 10 μl of 0.1% SDS, and β-galactosidase activity estimated at 28 °C as described in ref. 19.

When the 96-well microplate format was used, β-galactosidase activity assays were carried out as described in ref. 19 with modifications. R. capsulatus cultures were incubated overnight under diazotrophic conditions inside a glove box in a 96-well plate (black/clear Optilux™ flat bottom; BD Biosciences) covered with a transparent adhesive sealer. One hundred and twenty μl of each culture were transferred to a 96-well microplate containing 100 μl of Z-Buffer in each well20, then supplemented with 25 μl of 4-methylumbelliferone (β-D-galacto-pyranoside (MUG; 1 mg/ml stock solution in dimethyl sulfoxide), and incubated at room temperature for 2 h in darkness. MUG hydrolysis by β-galactosidase was quantified by fluorescence emission at 445 nm (372 nm excitation wavelength) in a Genios Pro (Tecan) microplate fluorometer.

Construction of random mutagenesis libraries. Random mutagenesis of nifH was carried out by Error-Prone PCR using PCR GeneMorph® II Random Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. Reaction mixtures contained 1 μl DNA template (0.4 ng of prRB529 including 0.1 ng of nifH), 5 μl of 10X Mutazyme II reaction buffer, 1 μl of 40 mM dNTP mix (200 μM each final), 0.5 μl of primer mix (250 ng/μl of each primer), 1 μl of Mutazyme II DNA polymerase (2.5 U/μl), and 41.5 μl of H2O. Primers P19 and P20 were used to amplify nifH gene by Mutazyme II DNA polymerase. PCR conditions used were: 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, and finished with an incubation at 72 °C for 10 min. Amplified DNA was digested with NdeI and XbaI, ligated into pRH8602, and introduced into E. coli DH5α competent cells (NEB, C29871) by heat shock to generate a expression library of nifH variants. On average, ~4 × 106 transformants were recovered per 100 ng DNA.

In vivo hydrogenase activity assays. Hydrogenase activity was measured by using a Clark-type hydrogen microelectrode (Unisense) with O2 as electron acceptor. When necessary, hydrogenase expression was induced by injecting 9 ml of H2 into 100 ml-capped vials containing 10 ml of R. capsulatus cultures and incubating under culture conditions for 6 h.

In vivo nitrogenase activity assays. To determine acetylene reduction activity in R. capsulatus cultures grown under diazotrophic conditions, 1-ml samples were transferred to 9-ml sealed vials with a 94% N2/6% acetylene gas phase and incubated at 30 °C in the light for 1 h. Ethylene formation was detected in 50μl samples withdrawn from the gas phase by using a Shimadzu GC-2014 gas chromatographer equipped with a 9-ft long, 1/8-in diameter Porapak R column. In vivo nitrogenase activity units are defined as nmol ethylene formed per min per ml of culture at an OD600 equal to 1.

To determine H2 production in R. capsulatus cultures grown under diazotrophic conditions, 16-ml samples were transferred to 23-ml sealed vials with a 100% N2 atmosphere and incubated at 30 °C in the light for 48 h. H2 formation was detected in 250μl samples withdrawn from the gas phase by using a Shimadzu GC-8A gas chromatographer equipped with a 6-ft long, 1/8-in diameter Molecular Sieve column. Activity units are defined as nmol H2 formed per h in a culture at an OD600 equal to 1.

Protein methods. Protein concentration was determined by the bicinchoninic acid method (Pierce) with bovine serum albumin as the standard. For SDS-PAGE, cells from 1-ml culture samples were collected by centrifugation, resuspended in 2 × Laemmli sample buffer supplemented with 0.1 M dithiothreitol (to a concentration equivalent to an OD600 of 4), and electrophoresed in 12% acrylamide/bisacrylamide (29:1) gels. For immunoblot analysis proteins were transferred to nitrocellulose membranes for 40 min at 20 V using a Transfer-Blot® Semi-Dry system (Bio-Rad). Immunoblot analyses were carried out with antibodies raised against a 1:1 mixture of A. vinelandii and Rhodospirillum rubrum NifH proteins (1:2,500 dilution) or with antibodies raised against R. capsulatus NifDK (1:2,000 dilution; antibody kindly donated by Yves Jouanneau, CNRS, Grenoble). Secondary alkaline phosphatase-conjugated anti-rabbit antibodies (Sigma, A3687) were used at 1:5,000 dilution.

Flow cytometry. Cells from 50-ml R. capsulatus cultures under diazotrophic conditions inside a glove box were collected by centrifugation in Falcon tubes for 15 min at 4 °C, 4500 × g, resuspended in 5 ml PBS supplemented with 10% glycerol, and incubated for 30 min at 4 °C. Cells were then collected, resuspended in 1 ml of an 8:1:1 mixture of PBS, fluorescein di-β-D-galactopyranoside (FDG) and propidium iodide (PI), and incubated at 37 °C for 30 min to facilitate FDG entrance into the cells. FDG releases fluorescein when cleavage by β-galactosidase is detected. Cells were collected by centrifugation, resuspended in RCV medium supplemented with Tc, and analyzed in a FACS Vantage (sorter) flow cytometer using an argon ion laser to excite the fluorochrome (488 nm). Cells exhibiting high fluorescence levels were sorted and recovered in 96-well microplates containing YPS medium supplemented with Tc.

NifH 3-D models. 3-D model of R. capsulatus NifH and the V7 variant were generated by homology modeling (http://swissmodel.expasy.org/) using the A. vinelandii NifH structures as template (1NIP for wild-type NifH and 1G5P for NifH-V7, respectively). Both models yielded NifH homodimer with capability to ligate [4Fe-4S] clusters.

References
1. Hallenbeck, P. C. & Ghosh, D. Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol. 27, 287–297 (2009).
2. Hoffman, B. M., Lukyanov, D., Yang, Z. Y., Dean, D. R. & Seefeldt, L. C. Mechanism of nitrogen fixation by nitrogenase: the next stage. Chem. Rev. 114, 4041–4062 (2014).
3. Hunter, C. N., Daldal, F. C. T. M. & Beatty, J. T. In Advances in Photosynthesis and Respiration Vol. 28 (Springer, Dordrecht, The Netherlands, 2008).
4. Kranz, R. G. & Haselkorn, R. Anaerobic regulation of nitrogen-fixation genes in *Rhodopseudomonas capsulata*. Proc. Natl. Acad. Sci. USA. 83, 6803–6809 (1986).

5. Straat, H. et al. Complete genome sequence of the photosynthetic purple nonsulfur bacterium *Rhodobacter capsulatus* SB 1003. *J. Bacteriol.* 192, 3545–3546 (2010).

6. Scolnik, P. A. & Haselkorn, R. Activation of extra copies of genes coding for nitrogenase in *Rhodopseudomonas capsulata*. *Nature* 307, 289–292 (1984).

7. Masepohl, B. & Hallenbeck, P. C. Nitrogen and molybdenum control of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*. *Adv. Exp. Med. Biol.* 675, 49–70 (2010).

8. Vignais, P. M. & Billoud, B. Occurrence, classification, and biological function of hydrogenases: an overview. *Chem. Rev.* 107, 4206–4272 (2007).

9. Bulet, W. A. & LeComte, J. R. The nitrogen system from Azotobacter: two-enzyme requirement for *N*2 reduction, ATP-dependent H2-evolution, and ATP hydrolysis. *Proc. Natl. Acad. Sci. USA.* 56, 979–986 (1966).

10. Loveless, T. M. & Bishop, P. E. Identification of genes unique to Mo-independent nitrogenase systems in diverse diazotrophs. *Can. J. Microbiol.* 45, 312–317 (1999).

11. Vignais, P. M. & Colbeau, A. Molecular biology of microbial hydrogenases. *Curr. Issues Mol. Biol.* 6, 159–188 (2004).

12. Colbeau, A. et al. Organization of the genes necessary for hydrogenase expression in *Rhodobacter capsulatus*. Sequence analysis and identification of two hyp regulatory mutants. *Mol. Microbiol.* 8, 15–28 (1993).

13. Vignais, P. M.,elsen, S. & Colbeau, A. Transcriptional regulation of the uptake [NiFe]hydrogenase genes in *Rhodobacter capsulatus*. *Biochem. Soc. Trans.* 33, 28–32 (2005).

14. Durmowicz, M. C. & Maier, R. J. Roles of HoxX and HoxA in biosynthesis of hydrogenase in *Bradyrhizobium japonicum*. *J. Bacteriol.* 179, 3676–3682 (1997).

15. Buhrke, T., Lenz, O., Krauss, N. & Friedrich, B. Oxygen tolerance of the H2-sensing [NiFe] hydrogenase from *Rhodobacter capsulatus*. *J. Bacteriol.* 174, 4258–4264 (1992).

16. Colbeau, A. et al. *Rhodobacter capsulatus* HupR is involved in regulation of hydrogenase synthesis through the HupUV proteins. *Eur. J. Biochem.* 251, 65–71 (1998).

17. Dischert, W., Vignais, P. M. & Colbeau, A. The synthesis of *Rhodobacter capsulatus* HupSL hydrogenase is regulated by the two-component HupT/HupR system. *Mol. Microbiol.* 34, 995–1006 (1999).

18. Vidal-Aroca, F. et al. One-step high-throughput assay for quantitative detection of β-galactosidase activity in intact Gram-negative bacteria, yeast, and mammalian cells. *Biotechniques* 40, 433–440 (2006).

19. Wecker, M. S. & Seibert, M. S. The transcriptional regulation of *Rhodobacter capsulatus* hydrogenase activity by HupR. *J. Bacteriol.* 187, 1151–1152 (2005).

20. Liu, T., Li, X. & Zhou, Z. Improvement of hydrogen yield by hupR gene knock-out and nifA gene overexpression In *Rhodobacter sphaeroides* 6016. *Int. J. Hydrogen Energy* 35, 9603–9610 (2010).

21. Masukawa, H., Mochimagi, M. & Sakurai, H. Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photoelectrochemical hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. *Appl. Microbiol. Biotechnol.* 58, 618–624 (2002).

22. Zhu, R., Wang, D., Zhang, Y. & Li, J. Hydrogen production by divTGB hupL double mutant of *Rhodospirillum rubrum* under different light conditions. *Chinese Science Bulletin* 51, 2611–2618 (2006).

23. Kim, E., Lee, M., Kim, M. & Lee, J. Molecular hydrogen production by nitrogenase of *Rhodobacter sphaeroides* and by Fe-only hydrogenase of *Rhodospirillum rubrum*. *Int. J. Hydrogen Energy* 33, 11306–11312 (2008).

24. Rey, F. E., Heininger, Ë. K. & Harwood, C. S. Redirection of metabolism for biological hydrogen production. *Applied and environmental microbiology* 73, 1665–1671 (2007).

25. Wang, D., Zhang, Y., Welch, E., Li, J. & Roberts, G. P. Elimination of Rubisco alters the regulation of nitrogenase activity and increases hydrogen production in *Rhodospirillum rubrum*. *Int. J. Hydrogen Energy* 35, 7377–7385 (2010).

26. Veland, L., Berg, K. M., Storå, J. & Berg, C. H. Complete genome sequence of the photosynthetic purple nonsulfur bacterium *Chlamydomonas reinhardtii*. *Plant Physiol.* 122, 127–136 (2000).

27. Flanagan, L. A., Wright, J. J., Roesler, M. M., Moir, J. W. & Parkin, A. Re-engineering a NiFe hydrogenase to increase the H2 production bias while maintaining native levels of O2 tolerance. *Chem. Commun.* 52, 9133–9136 (2016).

28. Christiansen, J., Cash, V. L., Seefeldt, L. C. & Dean, D. R. Isolation and characterization of an acetylene-resistant nitrogenase. *J. Biol. Chem.* 275, 11459–11464 (2000).

29. Masukawa, H., Inoue, K., Sakurai, H., Wolk, C. P. & Hausinger, R. P. Site-directed mutagenesis of the *Anabaena* sp. strain PCC 7120 nitrogenase active site to increase photoelectrochemical hydrogen production. *Appl. Environ. Microbiol.* 76, 6741–6750 (2010).

30. Tacker, M. S. & Liu, D. R. Methods for the directed evolution of proteins. *Nat. Rev. Genet.* 16, 379–394 (2015).

31. Griswold, K. E. et al. Evolution of highly active enzymes by homology-independent recombination. *Proc. Natl. Acad. Sci. USA.* 102, 10082–10087 (2005).

32. Peters, J. W., Boyd, E. S., Hamilton, T. & Rubio, L. M. In *NiFe hydrogenase* (ed Moir, J. W. B.) 59–99 (Caister Academic Press, 2011).

33. Flynn, T. M. Accumulation of O2-tolerant phenotypes in H2-producing strains of *Chlamydomonas reinhardtii* by sequential applications of chemical mutagenesis and selection. *Int. J. Hydrogen Energy* 27, 1421–1430 (2002).

34. Seibert, M. Benson, D. K. & Flynn, T. M. Method and apparatus for rapid biohydrogen photoprototyping of microorganisms using a chemochromic sensor. US patent 6, 277,589 B1 (2001).

35. Katsuda, T., Ooshima, H., Azuma, M. & Kato, J. New detection method for hydrogen gas for screening hydrogen-producing microorganisms using water-soluble wilkinson’s catalyst derivative. *J. Biosci. Bioeng.* 102, 220–226 (2006).

36. Wecker, M. S. A. & Ghirardi, M. L. High-throughput biosensor discriminates between different algal H2-photoproducing strains. *Biotechnol. Bioeng.* 111, 1332–1340 (2014).

37. Wecker, M. S. A., Messer, J. E., Posewitz, M. C. & Ghirardi, M. L. Design of a new biosensor for algal H2 production based on the H2-sensing system of *Rhodobacter capsulatus*. *Int. J. Hydrogen Energy* 36, 11229–11237 (2011).

38. Miller, J. H. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press. *466* (1972).

39. Albareda, M. et al. Dual role of HupF in the biosynthesis of [NiFe] hydrogenase in *Rhizobium leguminosarum*. *BMC Microbiol.* 12, 256 (2012).

40. Smith, P. K. et al. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85 (1985).

41. Plovin, A., Alvarez, A. M., Ibañez, M., Molina, M. & Nombela, C. Use of fluorescein-di-beta-D-galactopyranoside (FDG) and CI2-FDG as substrates for beta-galactosidase detection by flow cytometry in animal, bacterial, and yeast cells. *Appl. Microbiol. Biotechnol.* 60, 4638–4641 (1999).

Acknowledgements
Flow cytometry was performed at the Flow Cytometry Unit of the Interdepartmental Investigation Service (Sldl)-Faculty of Medicine (Universidad Autónoma de Madrid). We thank Laura Molero Martín for help in the design
of the flow cytometry protocol. We thank Luis Fernández Pacios for developing the model of the V7 NifH variant. This work was supported by the European Research Council Starting Grant 205442 and by MINECO Grant BIO2014-59131-R.

Author Contributions
E.B. performed molecular biology, cellular biology, flow cytometry and biochemical assays. E.J.V. contributed biochemical assays. E.B. and L.M.R performed the experimental design and data analysis and wrote the paper. L.M.R initiated and directed this research.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Barahona, E. et al. Hydrogen overproducing nitrogenases obtained by random mutagenesis and high-throughput screening. Sci. Rep. 6, 38291; doi: 10.1038/srep38291 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2016
Corrigendum: Hydrogen overproducing nitrogenases obtained by random mutagenesis and high-throughput screening

Emma Barahona, Emilio Jiménez-Vicente & Luis M. Rubio

Scientific Reports 6:38291; doi: 10.1038/srep38291; published online 02 December 2016; updated on 24 February 2017

This Article contains a typographical error in the Conclusions section.

“In this work, we have re-engineered the natural H₂ sensing system of R. capsulatus and combined it with FACS for the high-throughput selection of nitrogenase variants with enhanced H₂ production that independently retain their N₂ fixation activity”.

should read:

“In this work, we have re-engineered the natural H₂ sensing system of R. capsulatus and combined it with FACS for the high-throughput selection of nitrogenase variants with enhanced H₂ production that might independently retain their N₂ fixation activity”.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017