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Improved hydrogen production by uptake hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* O.U.001

Gökhan Kars[a], *Ufuk Gündüz*[a,*], *Gabor Rakhely*[b], *Meral Yücel*[a], *İnci Eroğlu*[c], *Kornel L. Kovacs*[b]

[a]*Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey*
[b]*Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary*
[c]*Department of Chemical Engineering, Middle East Technical University, 06531 Ankara, Turkey*

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ABSTRACT

Rhodobacter sphaeroides O.U.001 is a purple non-sulfur bacterium producing hydrogen under photoheterotrophic conditions. Hydrogen is produced by Mo-nitrogenase enzyme and substantial amount of H_2 is reoxidized by a membrane-bound uptake hydrogenase in the wild type strain. To improve the hydrogen producing capacity of the cells, a suicide vector containing a gentamicin cassette in the *hup*SL genes was introduced into *R. sphaeroiodes* O.U.001 and the uptake hydrogenase genes were destroyed by site directed mutagenesis. The correct integration of the construct was confirmed by uptake hydrogenase activity measurement, PCR and subsequent sequence analysis. The wild type and the mutant cells showed similar growth patterns but the total volume of hydrogen gas evolved by the mutant was 20% higher than that of the wild type strain. This result demonstrated that the hydrogen produced by the nitrogenase was not consumed by uptake hydrogenase leading to higher hydrogen production.

1. Introduction

Biological hydrogen production is of great importance since biohydrogen is a clean and sustainable energy carrier. Among biological hydrogen production methods, photo-biological hydrogen production by purple non-sulfur bacteria such as *Rhodobacter sphaeroides* is a potential candidate. In *R. sphaeroides*, hydrogen is produced under anaerobic conditions in light, using different organic substrates as carbon and energy source through the action of molybdenum containing nitrogenase. The evolved H_2 is free from O_2 but accompanied with CO_2 due to the utilization of organic substrates [1]. The light serves as the energy source and electrons are provided from organic substrates such as glucose, malate and acetate. The H_2 production is mediated by Mo-nitrogenase such that one H_2 is produced at the expense of 4 ATP using two electrons:

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i. \quad (1)$$

In the absence of any substrate such as N_2 nitrogenase acts as ATP dependent hydrogenase and all the electrons are utilized for H_2 production increasing the efficiency of the process [2,3].

In *R. sphaeroides*, several metabolic pathways take role in the production and consumption of hydrogen as shown schematically in Fig. 1. The total hydrogen production is limited due to several metabolic events occurring in the cells such as the production of poly-3-hydroxybutyrate (PHB) or consumption of hydrogen by uptake hydrogenase. The membrane-bound uptake hydrogenase decreases the efficiency of H_2 production by catalyzing the conversion of molecular hydrogen to electrons and protons [4]. It was

*Corresponding author. Tel.: +90 312 2105183; fax: +90 312 2107976.
E-mail address: ufukg@metu.edu.tr (U. Gündüz).

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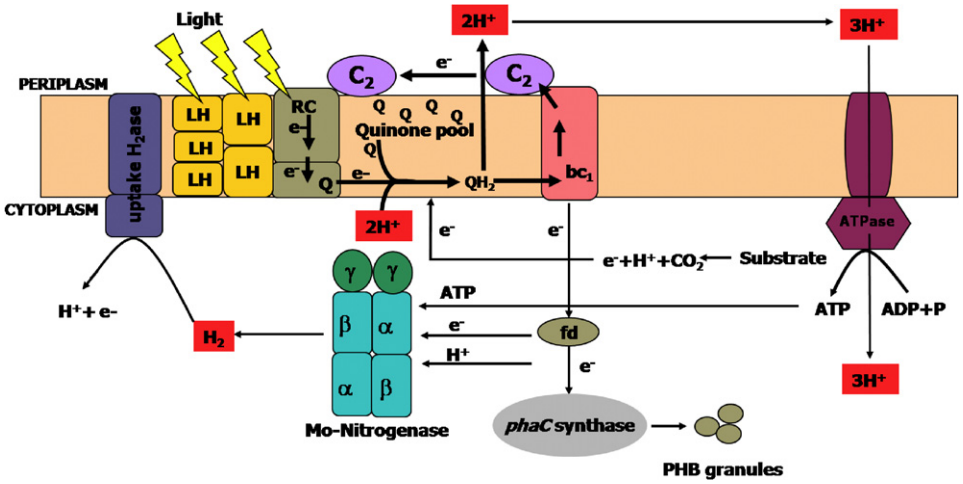

Fig. 1 – The schematic view of H_2 related pathways in *R. sphaeroides*.

reported that the inactivation of uptake hydrogenase resulted in total increase in hydrogen production [5–7]. In this study, the purple non-sulfur bacterium *R. sphaeroides* O.U.001 was manipulated such that the uptake hydrogenase was inactivated. Total hydrogen production, the rate of H_2 production and substrate conversion efficiency were improved in modified *hup*$^-$ *R. sphaeroides* O.U.001.

2. Materials and methods

2.1. The growth and culture conditions

Rhodobacter sphaeroides O.U.001 was grown in Biebl and Pfenning (1981) minimal medium [8] in which malate and glutamate (15 mM/2 mM) were used as carbon and nitrogen sources. The cultures were incubated at 30 °C in 60 ml bottles under the irradiance of 940 μE/m^2/s. If needed, the antibiotics were added at the following concentrations (mg/ml): gentamicin (Gm), 50; kanamycin (Km), 25. The *E. coli* strains were grown in Luria Broth (LB) media at 37 °C with antibiotics supplemented in the following concentrations (mg/ml): ampicillin (Amp) 100; kanamycin 25; streptomycin (Sm) 25 and tetracycline (Tet) 10. The 1.5% agar was used as a solidifying agent when solid media were needed for both *R. sphaeroides* and *E. coli*.

2.2. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study together with their relevant characteristics are listed in Table 1.

Table 1 – The plasmids and bacterial strains used in this work

Strain/ Plasmid	Characteristics/Genotype	Reference
E.coli		
XL1 Blue	*Δ*(*mcrA*)183, *Δ* (*mcrCB-hsdSMR-mrr*) 173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1 lac* [F' *proAB lacIqZ Δ* M15 Tn10 (Tet^r)]	Stratagene
S17-1(*λ*pir)	294 (*recA pro res mod*) Tp^r, Sm^r (pRP4-2-Tc::Mu-Km::Tn7), *λ* pir	[9]
R. sphaeroides		
O.U.001	Wild type	DSM 5864
GK1	*hup*$^-$	This work
Plasmids		
pK18*mobsacB*	Km^r, *sacB*, *RP4 oriT*, *ColE1 ori*	[10]
pBluescript SK(+)	Amp^r, cloning vector	Stratagene
p34-SGm	Gm^r cassette source	[11]
pGhup 1	3 kb *hup* gene fragment cloned into pBluescript SK (+)	This work
pGhup1Gm	Gm^r cassette inserted into pGhup1	This work
pGhup2Gm	Gm^r cassette with *hup* gene cloned into pK18*mobsacB*	This work

2.3. The construction of *Rhodobacter sphaeroides* O.U.001 *hup*$^-$ mutant strain

The suicide vector used for the inactivation of uptake hydrogenase was constructed as follows: A 3082 bp long *hup* gene fragment was amplified using *Pfu* DNA polymerase (Fermentas) and the following primer pairs; hupSL3 (left): 5′-TAACGGATTTCACCCCTTCC-3′, hupSL4 (right): 5′-GAATGGCGAG CAGTTTCTTC-3′. The PCR program was as follows: 2 min at 95 °C for pre-denaturation, 30 cycles of amplification step (1 min at 95 °C, 1 min at 50 °C and 7 min at 72 °C) followed by a final extension of 5 min at 72 °C. The PCR fragment was cloned into *Eco*RV cut pBluescript SK (+). The resulting construct (pGhup1) was analyzed by restriction enzyme digestion and sequence of the insert was confirmed. The Gm^r cassette was excised from the p34SGm after digestion with *Kpn*I and cloned into *Stu*I digested pGhup1 resulting in the construct pGhup1Gm. A 2.8 kb gene fragment containing 1 kb *hup* gene fragments flanking 0.8 kb Gm^r cassette was blunt end cloned into *Sma*I cut pK18*mobsac*B vector. The

constructed suicide vector (pGhup2Gm) was then transferred to *E. coli* S17-1(λpir) to deliver it to *R. sphaeroides* by conjugation. After conjugation, the cells were grown on B&P agar plates with Kanamycin (Km) and Gentamicin (Gm). The resistant colonies (single recombinants) were picked and grown in liquid B&P media for a few passages. Then the cells were spread on plates with 5% sucrose to select for the double recombinants. The colonies grown on sucrose containing plates were then replica plated on both Km containing plates and Gm containing plates to find the Km sensitive but Gm resistant *hup*$^-$ double recombinants. The presence of Gm^r cassette in the genomic DNA of double recombinants was also confirmed by colony PCR and sequencing of the product. The primers used for the colony PCR and sequence analysis were forward primer [5′-GCAGTCGCCCTAAAACAAAG-3′] and reverse primer [5′-AGTGCATCACTTCTTCCCGTA-3′]. The PCR program was the same as explained above except for the extension time which was 1 min at 72 °C.

The inactivation of uptake hydrogenase in the double recombinants was further confirmed by uptake hydrogenase activity assay which was performed using an artificial electron acceptor, benzyl viologen [12]. A 1.5 ml of anaerobically grown cells was centrifuged and dissolved in 1 ml of 20 mM phosphate buffer (pH = 7.0). 1 ml of phosphate buffer was added together with 100 μl of 40 mM BV. The mixture is first flushed with nitrogen gas to make anaerobic environment and then with hydrogen gas as a substrate of uptake hydrogenase for 5 min. This mixture was prepared in anaerobic cuvette and the kinetics of the reaction was measured the by spectrophotometer at the temperature at which the bacteria grow or at 60 °C. The color change due to reduction of benzyl viologen was recorded for certain time at the wavelength of 600 nm.

2.4. Hydrogen production and measurements

The hydrogen production with the *hup*$^-$ mutant strain was done using B&P medium with a special setup in which small scale bioreactors (60 ml bottles) were used. The evolved hydrogen gas was collected into water-filled tubes and the amount was recorded. The composition of the gas was analyzed by gas chromatography in a Hewlett–Packard Series II system with a thermal conductivity detector and a Propak Q column [13].

3. Results and discussion

3.1. The characterization of Rhodobacter sphaeroides O.U.001 *hup*$^-$ mutant strain

The uptake hydrogenase of *R. sphaeroides* O.U.001 was inactivated by Gm^r cassette insertion into the *hup*SL operon which includes the structural genes of this enzyme. The suicide vector (Fig. 2A) was delivered to *R. sphaeroides* O.U.001 and it was incorporated into the *hup* operon by homologous recombination. After first recombination event, the single recombinants were selected on Km and Gm containing plates. After a few passages in non-selective media, the cells are spread on plates with 5% sucrose and putative double recombinants were picked for further investigations. The *sac*B gene found in the pK18*mobsac*B vector encodes the levansucrase enzyme which utilizes sucrose and produce toxic compounds [10]. Therefore, the cells grown on sucrose containing plates are either double recombinants or wild type. To find the double recombinants the Gm^r fragment in the *hup* operon was PCR amplified (Fig. 2B) and the product was sequenced. The sequenced gene fragment showed 99% homology to the Gentamicin acetyltransferase 3.2 gene of cloning vector pUCP22 after a similarity search using the BLAST at the NCBI web page (http://www.ncbi.nlm.nih.gov/BLAST/). Moreover, the uptake hydrogenase assay showed that there is no color formation in the double recombinant containing cuvettes which means that the uptake hydrogenase enzyme was successfully inactivated in double recombinants (Fig. 2C).

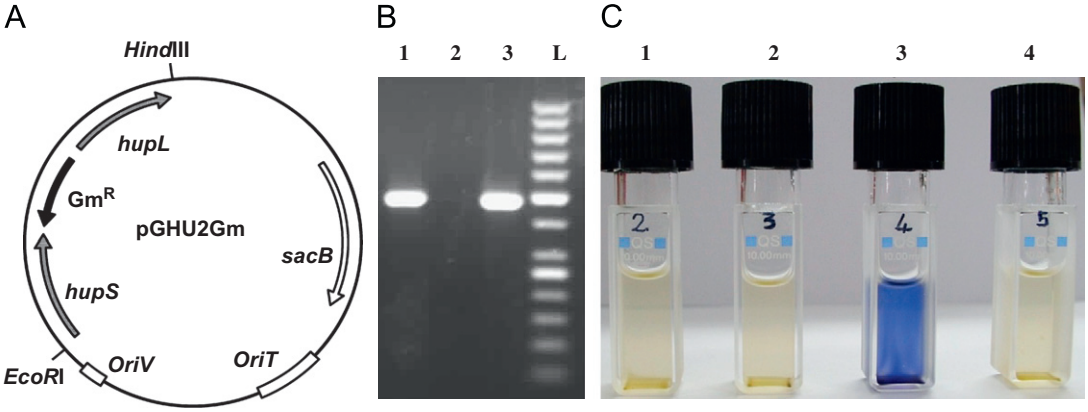

Fig. 2 – A suicide vector was constructed for the insertional inactivation of uptake hydrogenase genes (A). Agarose gel of PCR (B) by which the presence of Gm^r cassette was confirmed using the gDNA of *hup*$^-$ *R. sphaeroides* (1), gDNA of wild type cell (2) and the suicide vector (3) as a template. The uptake hydrogenase assay was performed (C) using *hup*$^-$ mutant *R. sphaeroides* (1), *hup*$^-$ mutant *R. sphaeroides* in the absence of hydrogen gas (negative control, 2), the wild type cells in the presence of H_2 gas (positive control, 3) and the wild type cells in the absence of H_2 gas (negative control, 4).

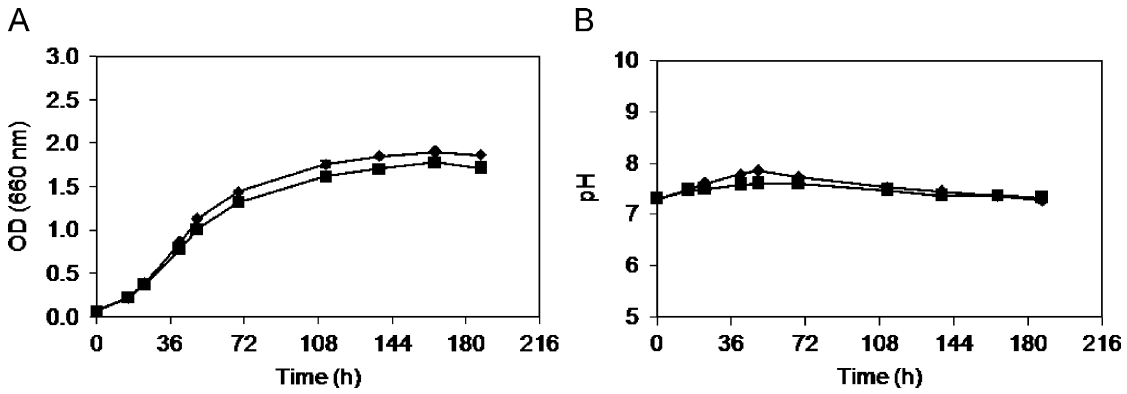

Fig. 3 – Absorbance (A) and pH (B) changes during the growth of wild type and mutant strain of *R. sphaeroides* O.U.001. Each value in the curves is the mean of three times replication with ± standard deviation. (■) *hup*⁻ mutant; (◆) wild type *R. sphaeroides* O.U.001.

3.2. *The growth of wild type and mutant strain of R. sphaeroides O.U.001*

The growth of mutant and wild type *R. sphaeroides* O.U.001 was monitored by measuring the absorbance at 660 nm at certain time intervals. The wild type cells reached relatively higher absorbance values (OD660 = 1.90 ± 0.05) compared to *hup*⁻ mutant strain (OD660 = 1.71 ± 0.06) (Fig. 3A). Moreover, the total cell dry weight measurements showed that the wild type cells reached higher cell masses (1.26 ± 0.10 g/L) while mutant cells reached lower cell masses (1.07 ± 0.15 g/L). According to the previous studies [14], the cells which cannot produce hydrogen obtained higher cell masses than the cells which produce considerable amount of hydrogen. Therefore, the results are in consistent with the previous ones. This phenomenon could be attributed to the total amount of energy and electron spent either to hydrogen production or cell materials which means that more energy and electrons are directed towards the hydrogen production in *hup*⁻ mutant cells while less amount of energy and electrons are consumed for hydrogen production in wild type cells.

The pH changes during the growth of mutant and wild type cells were monitored and illustrated in Fig. 3B. The initial pH was buffered to 7.0 and was not controlled during the growth. There was no considerable difference in the pH values of mutant and wild type cells and the pH values varied between 7.3 and 7.8 during the cultivation.

3.3. *Comparative hydrogen production with wild type and mutant strain of R. sphaeroides O.U.001 in photobioreactor*

The total hydrogen photoproduction by *hup*⁻ mutant and wild type *R. sphaeroides* O.U.001 was investigated under the nitrogenase repressed conditions in 60 ml bioreactors. Previously, it was shown that the inactivation of uptake hydrogenase resulted in increase in total hydrogen production in the cells producing hydrogen by nitrogenases (5–7). Similarly, significantly higher amount of hydrogen was accumulated in *hup*⁻ mutant *R. sphaeroides* O.U.001 when compared to wild type cells (Fig. 4). The total hydrogen accumulation in mutant cells was 20% higher than that in

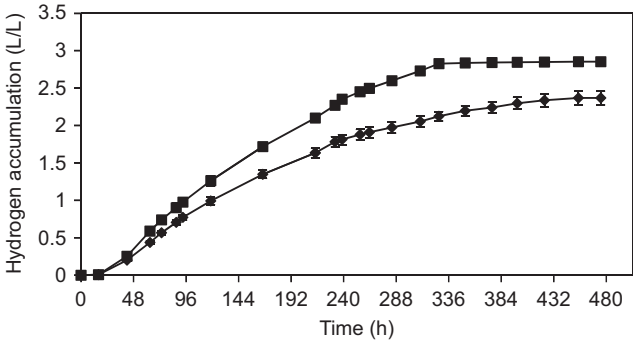

Fig. 4 – Total hydrogen production by wild type (◆) and *hup*⁻ mutant (■) *R. sphaeroides* O.U.001. Each value in the curves is the mean of three times replication with ± standard deviation.

wild type cells ($m = 2.85$ l H_2/l culture, $w = 2.36$ l H_2/l culture). According to the gas chromatography (GC) analysis, the hydrogen constituted 96–99% (v/v) of the overall gas. The average gas production rate of mutant cells was 9.2 ± 0.4 ml/l/h while that of wild type cells was 6.9 ± 0.5 ml/l/h which is calculated by dividing the total volume of gas produced by the volume of the culture and by the duration of gas production.

Another parameter for comparative analysis of hydrogen production is the substrate conversion efficiency which is calculated as the ratio of the actual amount of produced hydrogen to the theoretical amount. The substrate conversion efficiency of mutant cells was 85.2 ± 2% while that of wild type cells was 70.5 ± 3%. A substrate conversion efficiency of 35–57% for the malate was reported in the literature for *R. sphaeroides* (13). Hence, an efficient substrate conversion to hydrogen was achieved in *hup*⁻ mutant *R. sphaeroides* O.U.001.

To conclude, directed insertional inactivation of uptake hydrogenase significantly increased the total hydrogen production in *hup*⁻ mutant cells and it did not affect the growth of bacteria. The high substrate conversion efficiency demonstrated that more energy and reducing equivalents were directed towards the nitrogenase enzyme and therefore more hydrogen accumulation was achieved. Hence, the results are

promising for the genetic engineering of *R. sphaeroides* towards enhanced hydrogen production capacity.

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