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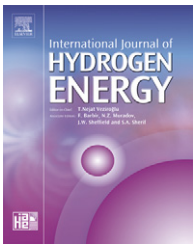

Molecular hydrogen production by nitrogenase of *Rhodobacter sphaeroides* and by Fe-only hydrogenase of *Rhodospirillum rubrum*

Eui-Jin Kim[a], *Moon-Kyu Lee*[a], *Mi-Sun Kim*[b], *Jeong K. Lee*[a,*]

[a]*Department of Life Science and Interdisciplinary Program of Integrated Biotechnology, Sogang University, Mapo, Shinsu 1, Seoul 121-742, Republic of Korea*

[b]*Biomass Research Team, Korea Institute of Energy Research, Daejeon 305-343, Republic of Korea*

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ABSTRACT

The genes coding for two PII-like proteins, GlnB and GlnK, which play key roles in repressing the nitrogenase expression in the presence of ammonium ion, were interrupted from the chromosome of *Rhodobacter sphaeroides*. The *glnB–glnK* mutant exhibits the less ammonium ion-mediated repression for nitrogenase compared with its parental strain, which results in more H_2 accumulation by the mutant under the conditions. *Rhodospirillum rubrum* produces H_2 by both nitrogenase and hydrogenase. *R. rubrum* containing the recombinant pRK415 with an insert of *hydC* coding for its own Fe-only hydrogenase showed twofold higher accumulation of H_2 in the presence of pyruvate under photoheterotrophic conditions, which was not observed in the absence of pyruvate. The same was true with *R. rubrum* containing the recombinant pRK415 cloned with *hydA* coding for Fe-only hydrogenase of *Clostridium acetobutylicum*. Thus, Fe-only hydrogenase requires pyruvate as an electron donor for the production of H_2.

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1. Introduction

The molecular hydrogen production by purple non-sulfur photosynthetic bacteria during photosynthetic growth is directly mediated by nitrogenase [1,2], which is regulated transcriptionally and post-transcriptionally by its own end product, ammonium ion [3–8]. Nitrogenase activity is inhibited when the dimer of NifH (dinitrogenase reductase) are ADP-ribosylated by DraT [4,5]. Two PII-like proteins, GlnB and GlnK, activate DraT in response to ammonium ion and darkness [6]. The expression of *nifHDK*, structural genes coding for nitrogenase, is also repressed in response to ammonium ion. The activity of NifA, a transcriptional activator for *nifHDK*, appears to be inactivated by GlnB and GlnK [6]. In addition, GlnB inhibits a sensor kinase NtrB under nitrogen-replete conditions, which results in de-phosphorylation of a transcriptional activator NtrC. The transcription of *nifA* is no longer activated as a result [6–8].

The electrons of molecular hydrogen can be used as a reductant for photoautotrophic growth by the membrane-bound H_2-uptake hydrogenase, which is classified as NiFe–hydrogenase [9]. Although NiFe-hydrogenase catalyzes the production or consumption of H_2 in the presence of a suitable electron donor or acceptor, respectively, its physiological function is an antagonist of nitrogenase in terms of H_2 accumulation [10–12]. The H_2 accumulation of purple non-sulfur photosynthetic bacteria is determined by the combined activities of nitrogenase and hydrogenase. The final level of

*Corresponding author. Tel.: +82 2 705 8459; fax: +82 2 704 3601.
E-mail address: jgklee@ccs.sogang.ac.kr (J.K. Lee).

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H_2 accumulation in *Rhodobacter sphaeroides* is affected by the H_2-evolving activity of nitrogenase and the H_2-consuming activity by uptake hydrogenase.

Previously, we isolated a purple non-sulfur photosynthetic bacterium from mud samples collected at the seashore of Korea, which showed higher production of H_2 in comparison with the laboratory strain, *R. sphaeroides* 2.4.1 [13]. The isolate, which was identified and deposited as *R. sphaeroides* KCTC 12085, was used as a wild type in this study. In our previous study, a double mutant HP1 which has mutations in H_2-uptake hydrogenase and PHB synthase was used for H_2 production, because the mutant cells showed increased level of H_2 accumulation in comparison with the wild-type cells [13]. Improvement of H_2 production by HP1 represents that the nitrogenase-mediated H_2 accumulation of *R. sphaeroides* under photoheterotrophic condition is directly reduced by the hydrogenase activity catalyzing H_2 uptake and indirectly by energy-demanding metabolic processes such as PHB formation.

Rhodospirillum rubrum is a purple non-sulfur photosynthetic bacterium, which produces molecular hydrogen by nitrogenase and hydrogenase [14,15]. In addition to the nitrogenase-mediated photoheterotrophic H_2 production, it was known that *R. rubrum* produces H_2 by formate–hydrogen lyase complex in the dark fermentative growth conditions [16]. Hydrogenase can be classified according to its metal cofactor(s). Although NiFe–hydrogenase catalyzes the production or consumption of H_2 in relation to the equilibrium of electron donor and acceptor, the activity of Fe-only hydrogenase, including HydC of *R. rubrum* and HydA of *Clostridium acetobutylicum*, is toward to the evolution of H_2 [17–20]. HydC of *R. rubrum* has a H-cluster domain, the active site for H_2 evolution, and showed 44% similarity with HydA of *C. acetobutylicum*.

In this work we disrupted two PII-like proteins, GlnB and GlnK, to de-repress the nitrogenase expression of *R. sphaeroides* in the presence of ammonium ion. The resulting mutant exhibits the less repression for nitrogenase expression and the more H_2 accumulation in the presence of ammonium ion. We also characterized Fe-only hydrogenase of *R. rubrum* and *C. acetobutylicum*, and found that Fe-only hydrogenase requires pyruvate as an electron donor for the production of H_2.

2. Materials and methods

2.1. Bacterial strains and growth conditions

R. sphaeroides KCTC 12085 (KCTC: the Korean Collection for Type Cultures) [13], *R. sphaeroides* 2.4.1 and *R. rubrum* UR1 were used as wild-type strains. *R. sphaeroides* was grown aerobically or photoheterotrophically at 28 °C in Sistrom's succinate minimal medium [21] as described previously [22]. *R. rubrum* was grown aerobically or photoheterotrophically at 28 °C in MN minimal medium as described previously [23]. Light intensity for photoheterotrophic growth was measured at the surface of culture vessels with a photometer (Li-Cor, Inc., USA) as described previously [22]. Light intensity was adjusted to 10 W/m^2. Modified Sistrom's medium containing DL-malate (30 mM) and L-glutamate (7 mM) was used for the H_2 accumulation of *R. sphaeroides* [13]. *R. rubrum* was grown in MG medium [23] supplemented with $NiCl_2$, Na_2SeO_3, and Na_2WO_4. When necessary, sodium pyruvate (30 mM) was added to the medium. Cell growth was monitored with a Klett–Summerson colorimeter (Manostat, USA) equipped with a KS-66 filter. *Escherichia coli* was grown at 37 °C in Luria–Bertani (LB) medium. When appropriate, kanamycin (Km) was added to the culture at the final concentration of 25 and 12.5 μg/ml for *R. sphaeroides* and *R. rubrum*, respectively. Tetracycline was used at 1 μg/ml for both *R. sphaeroides* and *R. rubrum* [13,24].

2.2. DNA manipulations and construction of mutant strains

All strains and plasmids used in this study are listed in Table 1. The nucleotide sequence of *R. sphaeroides* 2.4.1 was obtained from the genome site at http://mmg.uth.tmc.edu/sphaeroides/. Genes of *R. sphaeroides* KCTC 12085 were disrupted through homologous recombination using appropriate DNA of *R. sphaeroides* 2.4.1 which had been cloned into pLO1 as described previously [13,25–27]. Deletion of genes on the chromosome of recombinants was confirmed by genomic Southern hybridization analysis [28]. pMFH1, a plasmid harboring *hydA* coding for Fe-only hydrogenase of *C. acetobutylicum* was obtained from Dr. Soucaille [19], and the 1.7-kB DNA fragment containing *hydA* was cloned in the same orientation as *lac/tet* promoter of pRK415 [29] to generate pRKhydA. The nucleotide sequence of *R. rubrum* UR1 was

Table 1 – Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristic(s)	Source of reference
Strains		
E. coli		
S17-1	C600::RP-4 2-(Tc::Mu) *thi pro hsdR hsdM*$^+$ *recA*	[30]
R. sphaeroides		
2.4.1	Wild type	Sistrom [21]
KCTC 12085	Natural isolate, wild type	[13]
HP1	KCTC 12085 derivative Δ(*hupSL phbC*)	[13]
HP1ΔGlnBK	HP1 derivative Δ(*glnB glnK*)	[25]
R. rubrum		
UR1	Wild type	Roberts [23]
Plasmids		
pLO1	Km^r *sacB*$^+$, RP4 *oriT*, ColE1 *ori*	[26,27]
pRK415	Tc^r *lac*$^+$ IncP1 *ori*	[29]
pMFH1	pUC18 + *hydA*	Soucaille [19]
pRKhydA	pRK415 + 1.7 kb DNA fragment harboring *hydA*	This study
pRKhydC	pRK415 + 1.6 kb DNA fragment harboring *hydC*	This study

obtained from the genome site at http://genome.ornl.gov/microbial/rrub/. The DNA fragment containing *hydC* (RruA0310) coding for Fe-only hydrogenase of *R. rubrum* was PCR amplified and cloned in the same orientation as *lac/tet* promoter of pRK415 to generate pRKhydC. Plasmids were mobilized from *E. coli* S17-1 into *R. sphaeroides* or *R. rubrum* through conjugation as described previously [24,30].

2.3. Determination of nitrogenase activity

Nitrogenase activity was measured by the acetylene reduction method as described previously [13,31]. Ethylene was analyzed using a GC-17A gas chromatograph equipped with a flame ionization detector and an 80/100 mesh Porapak R column (Alltech, Korea). Nitrogen was used as a carrier gas.

2.4. Measurement of hydrogen gas

R. sphaeroides was inoculated into the modified Sistrom's medium for H_2 evolution in the homemade serum bottle, followed by gassing with argon as described previously [13]. *R. rubrum* was inoculated into the modified MG medium for H_2 evolution in the same bottle as described for *R. sphaeroides*. For photoheterotrophic growth, cells were contained in the side arm of the bottle, and the arm tube was stood in a tube rack in front of light box. For dark fermentative cell growth, the bottle was wrapped with aluminum foil. The gas phase (200–300 μl) was withdrawn intermittently during cell growth and analyzed for H_2 by GC-17A gas chromatograph (Shimadzu, Japan) equipped with a thermal conductivity detector and a column containing 60/80 mesh Molecular Sieve 5A (Supelco, USA). Argon was used as a carrier gas. The analyses of H_2 accumulation in this work were performed at least three separate times and the data presented here were reproduced within standard deviations of 10–15%.

3. Results and discussion

3.1. Removal of PII-like proteins of *R. sphaeroides* resulted in increase of H_2 accumulation in the presence of ammonium ion

The genes coding for two PII-like proteins, GlnB and GlnK, were disrupted to eliminate the ammonium ion-mediated repressive effect on the expression of nitrogenase. The DNA encoding GlnB and GlnK were internally deleted from the chromosome of *R. sphaeroides* HP1. Single mutations in either *glnB* or *glnK* resulted in the same ammonium ion-mediated repression for the nitrogenase expression as its parental strain HP1 (data not shown). The *glnB–glnK* double mutant HP1ΔGlnBK was grown photoheterotrophically in the presence of increasing concentration of ammonium salt. When the cells are grown exponentially (~100 KU), cells were briefly washed with the fresh modified Sistrom's medium containing no ammonium salts, followed by measurement of nitrogenase activity. The resulting activity should reflect the expression level of nitrogenase. The nitrogenase activity of HP1ΔGlnBK was not different from that of its parental strain HP1 when the cells had been grown without ammonium salts (Fig. 1A). Both cells showed the decreased level of nitrogenase as they had been grown in the presence of increasing concentrations of ammonium salt. However, HP1ΔGlnBK showed higher nitrogenase activities compared with its parental strain HP1. Approximately 20% of nitrogenase activity was still detected with HP1ΔGlnBK grown in the presence of 2 mM ammonium salt. No such activity was observed with HP1 that had been grown in the same conditions. Thus, the *glnB–glnK* double mutant HP1ΔGlnBK exhibits the less ammonium ion-mediated repression for nitrogenase compared with its parental strain HP1.

Consistently, the H_2 accumulation of HP1ΔGlnBK was less affected by ammonium salt compared with its parent strain HP1 (Fig. 1B). HP1 and HP1ΔGlnBK revealed approximately

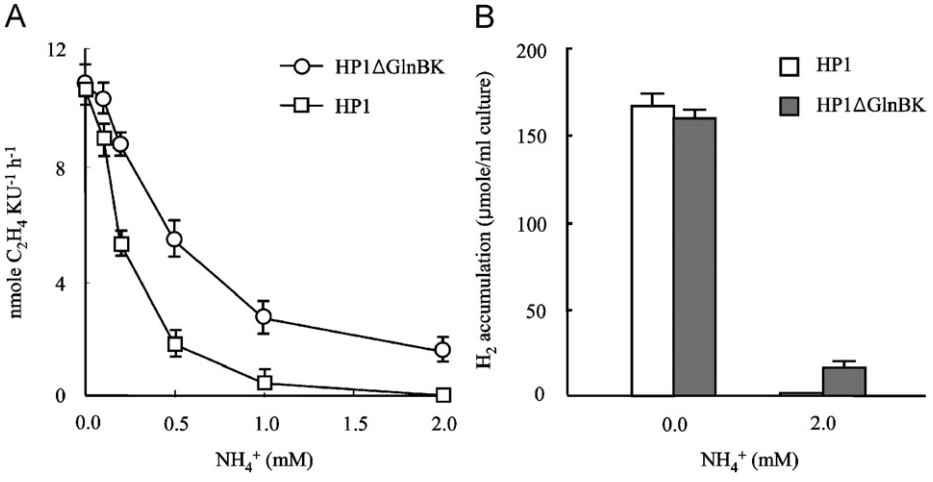

Fig. 1 – (A) Nitrogenase activities of *R. sphaeroides* HP1 (□) and HP1ΔGlnBK (○) which had been grown in the presence of increasing concentrations of ammonium salt. (B) Maximum H_2 accumulations of HP1 and HP1ΔGlnBK cells which had been grown in the absence or presence of ammonium salt (2 mM) under the photoheterotrophic conditions at 10 W/m^2.

160 μmole H_2 per ml of cell culture, when the cells had been grown without ammonium salt. HP1ΔGlnBK still accumulated 10% of H_2 when the cells were grown in the presence of 2 mM ammonium ions. However, no H_2 was detected from HP1 under the same growth conditions. It remains to be determined whether another PII-like protein(s) exists in *R. sphaeroides* KCTC 12085, since the ammonium ion-mediated repressive effect on the expression of nitrogenase still exists in *glnB–glnK* double mutant HP1ΔGlnBK.

3.2. *Photoheterotrophic H_2 production of R. rubrum was improved by elevating gene dosage of hydA and hydC coding for Fe-only hydrogenase of C. acetobutylicum and Fe-only hydrogenase of R. rubrum, respectively*

hydA and *hydC* were cloned in pRK415 to generate pRKhydA and pRKhydC. *R. rubrum* containing the recombinant plasmids were grown in the presence or absence of pyruvate (30 mM), and examined for the photoheterotrophic production of H_2 at the light intensity of 10 W/m^2. No difference in the maximum level of H_2 accumulation was observed between *R. rubrum* cells when the cells were grown without pyruvate (Fig. 2). However, the H_2 accumulation of *R. rubrum* (pRKhydA) and *R. rubrum* (pRKhydC) were increased up to nearly threefold when the cells were grown in the medium supplemented with pyruvate (Fig. 2). The H_2 production of *R. rubrum* containing pRK415 was also increased approximately twofold under the same condition, which may be resulted from the activation of chromosomally encoded Fe-only hydrogenase of *R. rubrum* (Fig. 2) since the nitrogenase expression of *R. rubrum* was not affected by the presence of pyruvate (data not shown). The level of H_2 accumulation of *R. rubrum* (pRKhydA) and *R. rubrum* (pRKhydC) were not different from that of *R. rubrum* containing pRK415 during dark fermentative growth (Table 2). Moreover, no H_2 accumulation was observed after the treatment of sodium hypophosphite (2 mM), a specific inhibitor for pyruvate–formate lyase (Table 2). Formate, which can be generated from pyruvate via an activity of pyruvate–formate lyase [16] can be used as a substrate for the hydrogen-evolving formate–hydrogen lyase. Thus, the H_2 accumulation of *R. rubrum* during dark fermentative growth is provided by pyruvate–formate lyase and formate–hydrogen lyase, and the Fe-only hydrogenase is not working under the conditions. Taken together, the results also indicate that Fe-only hydrogenase requires pyruvate as an electron donor for the production of H_2 in *R. rubrum*.

Interestingly, the slight reduction of H_2 accumulation was still observed when hypophosphite was treated to the photoheterotrophically grown cells (Fig. 3). The results indicate that the H_2 accumulation of *R. rubrum* (pRKhydA) and *R. rubrum* (pRKhydC) during photohetrotrophic growth was mediated by the elevated level of Fe-only hydrogenase although the pyruvate formate lyase and formate–hydrogen lyase are still active under both fermentative and photohetrotrophic growth conditions.

It remains to be determined how pyruvate acts as an electron donor for the production of H_2. However, the same H_2 accumulation with the Fe-only hydrogenase of *C. acetobuty-*

Table 2 – Effect of hypophosphite on the H_2 accumulation of *R. rubrum* harboring pRK415, pRKhydA, and pRKhydC under the dark fermentative growth conditions

Strains	Hydrogen accumulation (μ mole H_2/ml culture)	
	Without hypophosphite	With hypophosphite
R. rubrum UR1 (pRK415)	19.3 ± 0.7	ND[a]
R. rubrum UR1 (pRKhydA)	19.1 ± 0.8	ND
R. rubrum UR1 (pRKhydC)	18.5 ± 1.5	ND

[a] Not detectable.

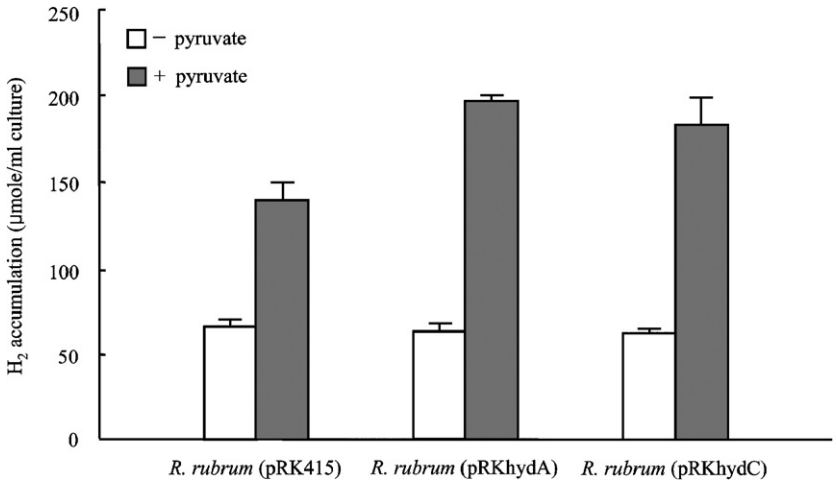

Fig. 2 – Maximum H_2 accumulations of *R. rubrum* containing pRK415, pRKhydA, and pRKhydC. Cells were grown in the absence or presence of pyruvate (30 mM) under the photoheterotrophic conditions at 10 W/m^2.

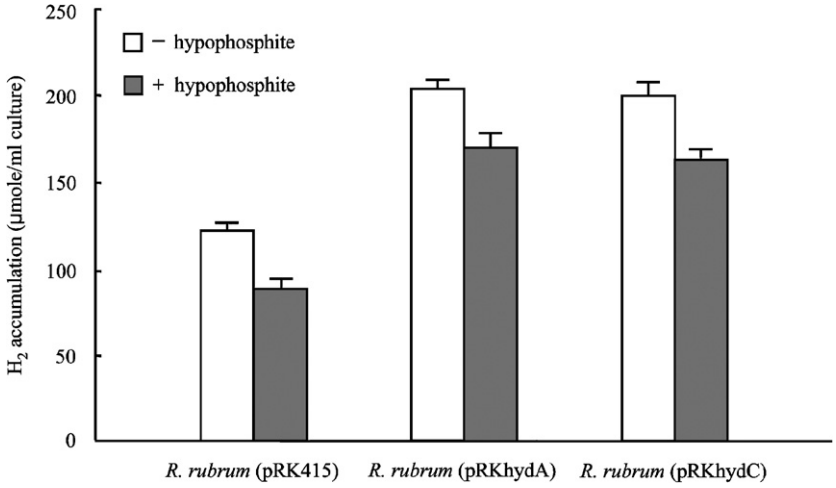

Fig. 3 – Maximum H_2 accumulations of *R. rubrum* containing pRK415, pRKhydA, and pRKhydC. Cells were grown in the absence or presence of sodium hypophosphite (2 mM) under the photoheterotrophic conditions at 10 W/m^2.

licum as that of *R. rubrum* under the conditions examined in this work suggest the presence of a common way to get electrons from pyruvate to the Fe-only hydrogenase in both bacteria. A pyruvate:ferredoxin oxidoreductase may be a plausible enzyme to provide electrons with Fe-only hydrogenase through the electron transfer via ferredoxin as proposed previously [32].

4. Concluding remarks

1. Mutations in both GlnB and GlnK exhibit the less ammonium ion-mediated repression for nitrogenase compared with its parental strain, which results in more H_2 accumulation by the mutant under the conditions.
2. Pyruvate–formate lyase and formate–hydrogen lyase are active under both fermentative and photohetrotrophic growth conditions.
3. Fe-only hydrogenase requires pyruvate as an electron donor for the production of H_2 in *R. rubrum*.

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