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The photoheterotrophic H_2 evolution of *Rhodobacter sphaeroides* is enhanced in the presence of ethanol

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ABSTRACT

Rhodobacter sphaeroides evolves H_2 by nitrogenase under photoheterotrophic growth conditions. The maximum accumulation level of H_2 increases by up to 60% when the culture medium, in which succinate and ammonium ion are the major carbon and nitrogen sources, is supplemented with ethanol at 0.5% (vol/vol). Such an effect by ethanol is not observed when ammonium ion is omitted from the medium. Ethanol is not used as a carbon source under the conditions examined, and the utilization of succinate and ammonium ion by cell is not affected by ethanol. The nitrogenase activity in the presence of ethanol, which is approximately 60% higher compared with that determined in its absence, is regulated at the level of *nif* transcription since the similar increase in the β-galactosidase activity of *nifH-lacZ* fusion was reproducibly observed under the same conditions. Interestingly, *nifA* transcription also increased similarly in the presence of ethanol. Neither *glnB* nor *glnK* interruptions abolished the ethanol-mediated enhancement of H_2 evolution. Thus, the presence of ethanol results in the enhancement of nitrogenase expression of *R. sphaeroides* through the increase of NifA expression in a manner independent of GlnB and GlnK.

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1. Introduction

The molecular H_2 is evolved by molybdenum–iron nitrogenase of *Rhodobacter sphaeroides* grown photoheterotrophically, which is usually observed after the depletion of ammonium ion in culture medium. Although the bacterium contains Ni–Fe hydrogenase, its activity is mainly involved in the uptake of H_2 [1–3]. Nitrogenase not only fixes molecular N_2 to form ammonium ion but also reduces H^+ simultaneously to form H_2 [4]. Eight electrons and sixteen ATPs are required to fix a N_2 with the generation of two ammonium ions and a H_2 [4]. However, nitrogenase can exclusively generate H_2 in the absence of N_2 [5].

Nitrogenase is a multi-subunit enzyme which consists of a reductase (NifH) and a catalytic complex (NifD and NifK). Electrons from ferredoxin or flavodoxin are transferred to Fe–S cluster of NifH, and its redox potential is lowered by ATP hydrolysis to reduce the Fe–Mo cofactor of NifDK, where the N_2 fixation and H^+ reduction take place [4]. Nitrogenase activity is inhibited by its product ammonium ion, which is mediated through the addition of ADP-ribosyl moiety to NifH by DraT [6,7]. PII proteins such as GlnB and GlnK activate DraT in response to ammonium ion [8–11]. The ADP-ribosyl moiety is then removed from NifH by DraG when ammonium ion is lowered [7]. Nitrogenase genes *nifH*, *nifD*, and *nifK* are organized

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in an operon and its transcription is activated by NifA [8–10]. GlnD is a bifunctional uridylyltransferase and uridylyl-removing enzyme [8,9]. It uridylylates PII proteins in the absence of ammonium ion, and the uridylylated PII activates NifA [8,9]. The transcription of NifA is in turn regulated by a two-component system of sensor kinase NtrB and response regulator NtrC [8–10]. GlnB inhibits NtrB under nitrogen-replete conditions, which results in de-phosphorylation of NtrC. Then, *nifA* transcription is no longer activated [8–10].

R. sphaeroides has been used as a bacterium to study the nitrogenase-mediated evolution of H_2 under the illuminated growth conditions [12–23]. Since the H_2 accumulation under photoheterotrophic conditions was elevated by the interruption of uptake hydrogenase [18], H_2 appears to be taken up by the uptake hydrogenase under the conditions, forming a futile cycle. An increase in H_2 accumulation was also observed when the energy for the formation of a reserve material poly-β-hydroxybutyrate (PHB) is saved by the mutation of PHB synthase [18,19]. Elimination of the most peripheral B800-850 light-harvesting (LH) complex under the light intensity saturated for cell growth resulted in the increase of H_2 accumulation [20]. The result was not due to the decrease of self-shade but due to the increase of energy save by the lack of the B800-850 LH complex formation, since the increase of light intensity from 10 to 100 W/m^2 did not further elevate the H_2 accumulation of the mutant and wild type [20]. However, the B875 LH complex was found essential for efficient light harvesting to get the H_2 evolution of wild type [20].

R. sphaeroides was further rendered to produce H_2 even in the dark by mobilization of the genes coding for pyruvate-formate lyase (PFL) and formate-hydrogen lyase (FHL) complex of *Rhodospirillum rubrum* [21]. The FHL was found to evolve H_2 even under photoheterotrophic condition, since approximately half the increment in photoheterotrophic H_2 accumulation by the recombinant *R. sphaeroides* harboring the plasmid carrying PFL and FHL was abolished by hypophosphite, an inhibitor of PFL. A putative FeFe-hydrogenase of *R. rubrum* was also shown to increase the H_2 accumulation when the coding gene was maintained *in trans* either in *R. rubrum* [22] or in *R. sphaeroides* [21]. The assembly of the active site H-cluster of FeFe-hydrogenase requires the concerted activities of the maturation proteins [24,25]. However, such maturation proteins are not found in *R. rubrum* and *R. sphaeroides*. It remains to determine how the FeFe-hydrogenase of *R. rubrum* shows the H_2 evolution activity in these bacteria.

Recently, we found that the addition of ethanol into the *R. sphaeroides* culture at the level less than 1% (vol/vol) increased the photoheterotrophic H_2 accumulation by up to 60% compared with wild type. The tolerance of *Escherichia coli* to ethanol at sub-lethal dose involves the changes in metabolic activities to enhance the degradation and assimilation of ethanol [26]. However, ethanol is not utilized by *R. sphaeroides* under the conditions. Nitrogenase activity was increased in the presence of ethanol, and the transcription of *nifHDK* as well as *nifA* was elevated under the conditions. The increase of H_2 accumulation in the presence of ethanol, which was not observed in ammonium ion-free medium, was still observed with *glnB* mutant and *glnK* mutant. Thus, the ethanol addition to the culture of *R. sphaeroides* results in the enhancement of *nifA* expression and consequent elevation of *nifHDK* transcription in a way independent of GlnB and GlnK.

2. Materials and methods

2.1. Bacterial strains and growth conditions

R. sphaeroides 2.4.1 was used as a wild-type strain. *R. sphaeroides* was grown aerobically or photoheterotrophically at 28 °C in Sistrom's succinate minimal medium (Sis medium) [27] as described previously [28]; the medium contains succinate (34 mM), ammonium sulfate (3.8 mM), L-glutamate (0.7 mM), and L-aspartate (0.3 mM) as major carbon and nitrogen sources. Light intensity for photoheterotrophic growth was measured as described previously [28], and was adjusted to 10 W/m^2. Cell growth was monitored with a Klett-Summerson colorimeter (Manostat, USA) equipped with a KS-66 filter. *E. coli* was grown at 37 °C in Luria–Bertani (LB) medium. Antibiotics for *R. sphaeroides* and *E. coli* cultures were added as indicated previously [20].

2.2. Construction of nifH-lacZ and nifA-lacZ fusion plasmids, and measurement of β-galactosidase activity

A 1184-bp DNA fragment extending from the 969-bp upstream from the initiation codon of NifH to its 215-bp downstream was transcriptionally fused to *lacZ* [29] on IncQ plasmid [30] to generate pCFnifH-lacZ. Likewise, a 546-bp DNA fragment extending from the 398-bp upstream from the initiation codon of NifA to its 148-bp downstream was transcriptionally fused to *lacZ* [29] on IncQ plasmid [30] to generate pCFnifA-lacZ. Both plasmids contain a transcription–translation stop Ω DNA (Sm^r/Sp^r) [31] at the border between the vector and *R. sphaeroides* DNA. The resulting recombinant plasmids were mobilized from *E. coli* S17-1 into *R. sphaeroides* by conjugation as described previously [32]. β-Galactosidase activity (Miller units) of the cells containing pCFnifH-lacZ and pCFnifA-lacZ was determined with o-nitrophenyl-β-D-galactoside as described previously [33].

2.3. Construction of glnB mutant and glnK mutant

A 276-bp DNA fragment extending from 15th amino acid residue to 107th residue of GlnK was deleted from approximately 1.7-kb *Sal*I-*Xho*I DNA spanning *glnK*. The resulting DNA fragment was cloned into pLO1 [34] to generate pLOΔglnK. Likewise, 255-bp DNA fragment extending from 4th amino acid residue to 89th residue of GlnB was deleted from approximately 0.9-kb *Sac*I-*Sal*I DNA spanning *glnB*. The resulting DNA fragment was cloned into pLO1 [34] to generate pLOΔglnB. The plasmids were mobilized into *R. sphaeroides*, and the single crossover exconjugant (Km^r) was isolated and subjected to segregation to double crossover (*glnK* mutant and *glnB* mutant: Km^s) on Sis medium agar plate containing sucrose (15%).

2.4. Measurement of hydrogen gas and nitrogenase activity

The H_2 evolved by *R. sphaeroides* under photoheterotrophic conditions was determined as described previously [35] with

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