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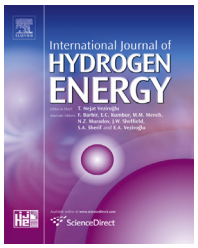

Improved ammonium tolerance and hydrogen production in *nifA* mutant strains of *Rhodopseudomonas palustris*

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ABSTRACT

Photosynthetic bacteria can utilize organic matter from wastewater to produce hydrogen gas, and the application of this process is an environment-friendly approach to produce energy. However, photo-fermentative hydrogen production can be inhibited by ammonium, which exerts an inhibitory role on the expression and activity of nitrogenase, the pivotal enzyme in hydrogen production. In this study, we constructed mutant strains of *Rhodopseudomonas palustris* by manipulating of nitrogen-regulatory genes. Photo-fermentative hydrogen production assay showed the ammonium concentration that *nifA draT2* and *nifA glnK2* double mutant strains can tolerate increased by 25 and 10 folds, respectively, compared with wild-type strain, though hydrogen yield decreased with the increase of ammonium concentration. Results indicate that manipulation of nitrogen-regulatory genes is an effective way to enhance the ammonium tolerance of photosynthetic bacteria and improve their hydrogen production.

Introduction

Several types of photosynthetic microorganisms, including green algae, purple non-sulfur bacteria, and cyanobacteria, have been considered for hydrogen production from renewable resources [1–5]. Purple non-sulfur bacteria use solar energy and organic carbon sources to produce hydrogen under anaerobic conditions. These bacteria can be applied to treat wastewater for hydrogen production, which is an economically sound process.

In purple non-sulfur bacteria, hydrogen is produced by nitrogenase. Under nitrogen-limiting conditions, nitrogenase is expressed and reduces protons into molecular hydrogen; this process has been developed for biological production of hydrogen fuel [6–8]. However, organic wastewater is often rich in ammonium or compounds that are broken down into ammonium [9]. The presence of ammonium strongly represses hydrogen production by regulating nitrogen fixation genes at three levels [10–15]. First, the expression of *nifA* gene, which codes for the transcription activator of other *nif* genes such as nitrogenase genes, is inhibited by ammonium. Second, the activity of NifA protein is regulated by PII-like proteins (GlnB and GlnK) based on N-status. Upon adding ammonium into a nitrogen-limiting culture, PII-like proteins can inhibit NifA activity. Third, the enzymatic activity of nitrogenase is regulated by two enzymes: dinitrogenase

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reductase ADP-ribosyltransferase (DraT, encoded by *draT*) and dinitrogenase reductase-activating glycohydrolase (DraG, encoded by *draG*). In the presence of ammonium, DraT modifies and inactivates nitrogenase via ADP-ribosylation, whereas in the absence of ammonium, DraG removes the ADP ribose group from nitrogenase to restore its activity. In addition, the activities of DraT and DraG are post-translationally regulated by PII-like proteins.

On the basis of regulatory mechanisms, a number of strains bringing mutations in *nifA* less sensitive to ammonium have been constructed to generate hydrogen from ammonium-containing medium. For example, a mutant of *Rhodopseudomonas palustris* containing a 48-nucleotide deletion mutation in *nifA* can produce hydrogen in a medium containing 2.5–10 mM ammonium [9]; a *Rhodobacter capsulatus* mutant containing A35V mutation in *nifA* produces 0.43 mL hydrogen/mL culture in the presence of 5 mM ammonium [16]; and a *Rhodobacter sphaeroides* strain harboring a vector expressing the *nifA* L62Q allele is considerably tolerant to increased ammonium concentration and releases hydrogen gas under 16 mM ammonium [17]. All these *nifA* mutants are more tolerant to ammonium than their parent strains, however, the ammonium tolerance of photosynthetic bacteria must be further improved because the organic wastewater used as hydrogen-producing medium usually contains high ammonium concentration [18]. Moreover, little information is about how to improve ammonium tolerance ability tolerance of photosynthetic bacteria efficiently. In the present study, *R. palustris nifA draT2* and *nifA glnK2* mutations were generated on the basis of the mechanism regulating nitrogenase and compared in terms of their hydrogen production capacity under various ammonium concentrations. Both strains displayed improved ammonium tolerance and produced hydrogen under high ammonium concentration.

Materials and methods

Bacterial strains and growth conditions

R. palustris strains were grown photoheterotrophically at 30 °C in Sistrom's medium [19] with ammonium sulfate or glutamate as nitrogen source. Light intensity for photoheterotrophic growth was set at 5000 lux. Cells were grown aerobically at 35 °C in ACY medium (Sistrom's medium supplemented with 5 g/L of casamino acids and 4 g/L of yeast extract). Acetate and butyrate were used as carbon sources for cell growth and hydrogen production. In addition, *Escherichia coli* cells were grown at 37 °C in Luria–Bertani (LB) medium supplemented with appropriate antibiotics as follows: 100 mg/L each of gentamicin (Gm) and kanamycin (Km) for *R. palustris*; and 100, 12, and 50 mg/L of ampicillin (Ap), Gm, and Km, respectively, for *E. coli*.

Plasmid and strain construction

Table 1 lists all the bacterial strains, plasmids, and primers described in this work. To construct *draT2* deletion mutants, the *draT2* gene was amplified with flanking regions through PCR by using the primers F1/R1 (Fig. 1a); the 2.3 kb PCR product was cloned into pBluKSP. A 1.0 kb TthlllI fragment was subsequently removed from the *draT2* gene. This construction was then cloned into pZJD29c, resulting in plasmid pLdraT2. For *glnK2* deletion mutation, the flanking regions of *glnK2* gene were amplified through PCR by using the primers F2/R2 and F3/R3 shown in Fig. 1c, and then cloned into the suicide vector pZJD29c, resulting in plasmid pLglnK2. For *nifA* mutation, DNA sequences of approximately 1 kb that flanked either side of the 48 bp nucleotides were amplified through PCR by using the primers F4/R4 and F5/R5 shown in Fig. 1e, and then cloned into the suicide vector pZJD29c, resulting in hybrid plasmid pLnifAQL.

Gene deletions were performed via homologous recombination. The *glnK2* and *draT2* mutation suicide vectors were separately transferred from *E. coli* S17-1 into *R. palustris* through conjugation. Single recombinants were selected on Sistrom's plates containing Gm. In addition, double recombinants were screened on ACY plates containing 8% sucrose (wt/vol), and the constructed mutant strains were CDT1 (Δ*draT2*) and CGK1 (Δ*glnK2*). Similarly, pLnifAQL was transferred into CGA009, CDT1, and CGK1, yielding CNF1 (*nifA**), CND2 (*nifA**Δ*draT2*), and CNG2 (*nifA**Δ*glnK2*). All these mutants were confirmed by colony PCR shown in Fig. 1. The primers used to confirm *draT2*, *glnK2*, and *nifA* mutants were F1/R1, F2/R3, and F6/R6, respectively.

Hydrogen production and analytical methods

The modified Sistrom's medium [19] containing ammonium sulfate (8 mM), acetate (20 mM), and butyrate (20 mM) were used to preculture the *R. palustris* strains. In the preculture, all strains were grown anaerobically in photo-incubators at 30 °C for 72 h. Cells were collected and inoculated into 10 mL of hydrogen-producing medium for hydrogen evolution in plastic syringes. Each test was conducted in triplicate. Biogas was collected in syringes and analyzed by gas chromatography [20]. Statistical differences between the means of experimental groups were determined using multiple Student's *t*-tests. A value of $P < 0.05$ indicates significant difference. Data points represent the means of three independent experiments.

Results and discussion

Characterization of mutant strains

The growth properties of *nifA* mutant strains CNF1 (*nifA**), CNG2 (*nifA**Δ*glnK2*), and CND2 (*nifA**Δ*draT2*) were examined in liquid medium containing acetate (20 mM) and butyrate (20 mM) as carbon sources and glutamate (8 mM) as nitrogen source. Fig. 2 shows the growth curves of each mutant strain and wild-type strain. The strains CNF1 and CND2, as well as the wild-type strain CGA009, demonstrated growth. The dry cell density of CNF1 and CND2 after 144 h was around 1.06 mg/mL, which was similar to that of the wild-type strain (1.05 mg/mL). This result indicates that the *draT2* and *nifA* gene mutations have no effect on the growth of *R. palustris*. Moreover, the *R. palustris nifA* and *glnK2* double-mutant strain CNG2 grew more slowly than the other strains. The dry cell density of