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Removal of the Effect of Ammonium on the Regulation of Nitrogenase Enzyme in *Rhodobacter Capsulatus* DSM1710 for Improved Hydrogen Production

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Abstract

Photofermentative biohydrogen production by purple non-sulfur (PNS) bacteria is a renewable and clean way of producing hydrogen. Hydrogen production by PNS bacteria, *Rhodobacter capsulatus*, is mediated mainly by nitrogenases, which primarily fix molecular nitrogen to ammonium and produce hydrogen as byproduct. The reaction catalyzed by nitrogenases requires a lot of energy. Hence, there is a complex regulation on nitrogenase enzyme complex, consequently, on hydrogen production. Whenever ammonium, which is the end product of nitrogen fixation reaction, is found in the environment, hydrogen production stops.

GlnB and GlnK proteins are the critical regulatory proteins in ammonium dependent regulation of the nitrogenase gene expression. In this study, the aim is to release the ammonium regulation on nitrogenase enzyme by inactivating *glnB* and *glnK* genes. For this purpose, relevant recombinant vectors were constructed; *R.capsulatus glnB*$^-$ strain was obtained. The double *R.capsulatus glnB*$^-$*glnK*$^-$ strain, able to produce hydrogen independent of ammonium concentration of the environment is to be obtained.

1 Introduction

Today, environmental pollution is one of the main problems of the world, mainly due to rapid industrialization and urbanization. Moreover, there is a high energy demand, whereas a limited amount of fossil fuels in the earth. Thus, increasing focus is being placed on clean and renewable energy. Hydrogen energy can be regarded as the fuel of future, since its commercial utilization is efficient and easy. Moreover, hydrogen can be completely recycled without CO_2 emission. Consequently, biohydrogen is a renewable and environmentally clean energy carrier [1].

The energy source of biohydrogen formation is the sun. Solar energy might be converted to hydrogen directly by some photosynthetic microorganisms or in two steps; first, the biomass is formed via photosynthetic organisms and second, the high energy compounds generated are converted to biohydrogen by fermentative organisms. In PNS bacteria, organic acids are utilized with light energy and nitrogenases in the photofermentation process. There are integrated biohydrogen production systems in which a dark fermentation process by fermentative bacteria is combined with a subsequent photofermentation process by PNS bacteria. Hence, the overall hydrogen yield can be enhanced to a great extent [1, 2]. One of

the projects which integrate dark fermentative and photofermentative hydrogen production is 'Hyvolution Project'. The effluent of the dark fermentation, which is rich in organic acids (i.e. acetate, lactate) and ammonium ions, is used in photofermentation. Biohydrogen is produced in both bioreactors.

Rhodobacter capsulatus is a PNS bacterium, which produces biohydrogen via photofermentation. It has two kinds of nitrogenase enzymes ([Fe-Mo] and [Fe-Fe]) that are responsible for hydrogen production. Nitrogenases normally reduce N_2 to ammonia, and also evolve hydrogen, particularly in the absence of N_2 gas. The overall reaction catalyzed by nitrogenase enzyme is [3]:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

This process is energetically expensive for the cell; therefore hydrogen production is strictly controlled by NH_4^+ both at the expression level and the enzyme activity level [4, 5]. If enough NH_4^+ is present in the environment, nitrogen fixation and hydrogen production completely cease [6]. This phenomenon decreases the hydrogen production efficiency of the integrated systems. The effluent of dark fermentation (which is used in photofermentation) has high ammonium concentrations enough to inhibit photofermentative hydrogen production. In order to increase the overall efficiency, ammonium insensitive hydrogen evolving strains must be used. The aim of the present study is to inactivate GlnB and GlnK proteins which are the key elements in ammonium dependent regulation of the nitrogenase gene expression. *R.capsulatus glnB⁻glnK⁻* strain will be capable of producing hydrogen irrespective of the ammonium concentration present in the medium.

2 Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

E.coli XL1 Blue (Stratagene) and *E.coli* S17-1 (λpir) [7] are *E.coli* strains used in this study. *Rhodobacter capsulatus* DSM1710 is the wild type strain of *R.capsulatus*. The cloning vector pBluescript SK (+) [*Amp*^{r}] (Stratagene) and the suicide vector pK18*mobsacB [Km*^{r}*, sacB, RP4 oriT, ColE1 ori]* [8] are the plasmids used in this study. The plasmids pGBBU, pGBBD, pGKBU, pGKBD, pGBSD, pGKSD, pGBSUD and pGKSUD were obtained in the present study.

Rhodobacter capsulatus DSM1710 was grown under continuous illumination at 30ºC in Biebl and Pfennig (BP) minimal medium [9], in which malate (7.5 mM) and glutamate (10.0 mM) were used as carbon and nitrogen sources, respectively. The vitamin solution (thiamin, niacin and biotin), trace elements solution and ferric citrate solution were added. *E.coli* strains were grown in Luria Broth (LB) medium at 37ºC with antibiotics in the following concentrations (μg/mL): ampicillin 100; kanamycin 25; and tetracycline 10.

2.2 General recombinant DNA techniques

Polymerase chain reaction (PCR), genomic DNA isolation, plasmid DNA isolation, restriction enzyme digestion, sticky or blunt end ligation, dephosphorylation of linearized plasmids, kinase treatment of PCR products, transformation of *E.coli* and conjugation were performed

according to the protocols described previously (Ausubel et. al., 1996) [10] and the supplier's instructions. Generuler 50 bp and 1kb DNA ladders (Fermentas) were used in agarose gel electrophoresis as DNA marker.

2.3 Sequence and Primer Design

Since the genome of *R.capsulatus* DSM1710 is not sequenced yet, the genome sequence of another strain (SB1003) was used for sequence information. 10 kb sequences including *glnB* and *glnK* genes were annotated. After obtaining exact sequences of DSM1710 by sequencing the PCR products, primers were designed to amplify both upstream and downstream fragments of *glnB* and *glnK* genes. Site directed mutagenesis (by deleting the internal fragments of the genes) was preferred to insertional mutagenesis, because the possible transfer of the antibiotic cassette present in the mutant organism to wild type (WT) organisms possesses a potential biosafety risk. Moreover, in order to keep the genes active, which are in the same operon with the targeted genes, the reading frame was kept as original.

2.4 Preparation of constructs for inactivation *glnB* and *glnK*

After PCR amplification of the upstream and downstream of *glnB* and *glnK* genes, the fragments were individually cloned into pBtSK (+), giving rise to pGBBU, pGBBD, pGKBU and pGKBD plasmids. Then, downstream fragments of *glnB* and *glnK* were excised from the relevant plasmids and cloned into the suicide vector (pK18*mobsacB*), yielding the pGBSD and pGKSD plasmids. By ligating these plasmids with upstream fragments excised from pGBBU and pGKBU, the final constructs for inactivation of *glnB* and *glnK* were obtained: pGBSUD and pGKSUD. The clones were checked by colony PCR. The plasmids were analyzed by restriction enzyme digestion and sequencing.

2.5 Gene transfer into *R.capsulatus* - Diparental mating

E.coli S17-1 cells containing the constructs are able to conjugate with *R.capsulatus* cells. Through conjugation between donor (*E.coli* S17-1) and recipient (*R.capsulatus* cells), constructs were transferred into *R.capsulatus* cells. The targeted genes were inactivated by means of homologous recombination between the construct and the genomic DNA of the cell. After the exchange of the genes by a two step homologous recombination, the targeted genes were inactivated, since the *glnB* and *glnK* genes in the constructs were deleted, nonfunctional genes.

2.6 Selection of mutants

Following conjugation, the cell mixture was spread onto kanamycin containing plates. Single recombinants which integrate the entire plasmid into the genomic DNA were selected based on kanamycin resistance. In order to enable cells to perform the second recombination, cells were grown in nonselective BP medium for a few passages. In the second recombination event, the entire plasmid (except homologous regions) was excised from the genomic DNA. Double recombinants were selected by using pK18*mobsacB* suicide vector, in which there is a *sacB* gene whose product converts sucrose to a toxic compound. Spreading the cells onto sucrose containing plates eliminated single recombinants. Thus, only the double recombinants could form colonies on sucrose containing plates, which can be either WT or

the expected mutant strain. The mutant strains were further selected and confirmed by the length of the PCR amplicon and the sequence results of the deletion sites.

3 Results and Discussion

Upstream and downstream fragments of *glnB* and *glnK* genes were amplified by unique primers and cloned into the pBtSK (+) cloning vector. Downstream fragments (~1 kb) were individually excised and ligated into the pK18*mobsacB* suicide vector (~5700 bps); giving pGBSD and pGKSD (~6700 bps) (Figure 1a, 2a). The upstream fragment of *glnB* and *glnK* were ligated to pGBSD and pGKSD, respectively. The final constructs obtained for the inactivation of targeted genes were pGBSUD (~7700 bps) (Figure 1b) and pGKSUD (~7700 bps) (Figure 2b). The validity of the constructs was confirmed by double restriction enzyme digestion to excise 1 kb insert (Figure 1c, 2c), colony PCR and sequence analysis (data not shown).

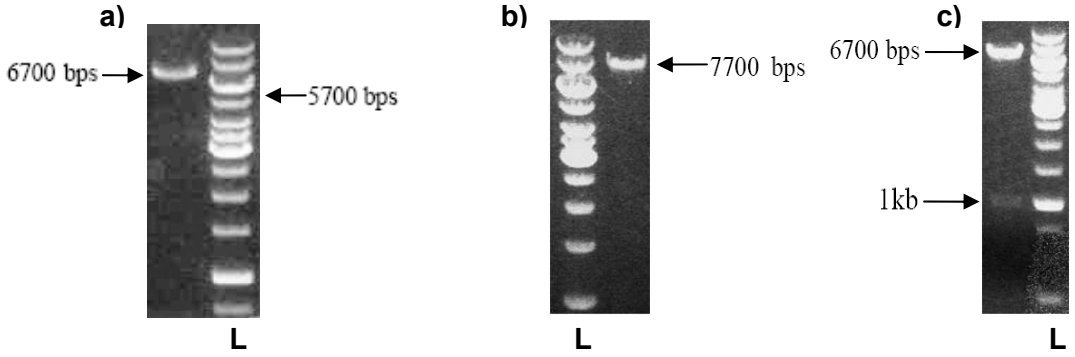

Figure 1: **a) Linearized pGBSD (~6700 bps) and the position of linearized pK18*mobsacB* in the ladder(~5700 bps), b) linearized pGBSUD (~7700 bps), c) double digestion of pGBSUD to excise the insert (~1 kb), L: 1 kb DNA ladder.**

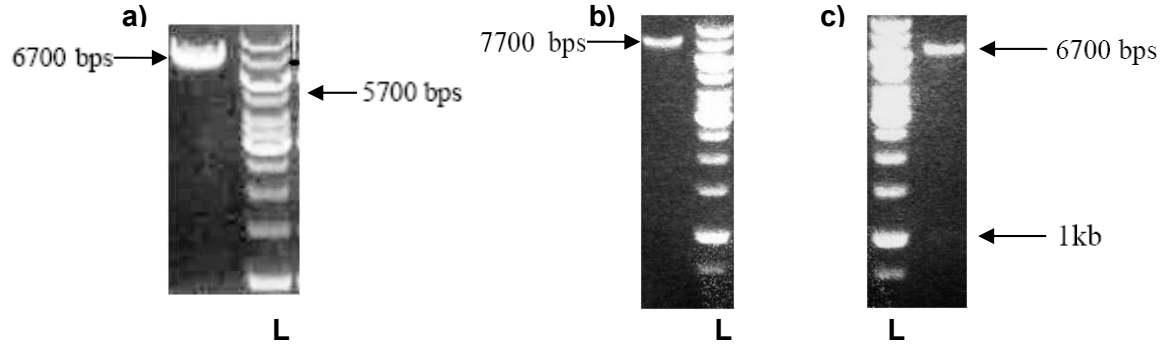

Figure 2: **a) Linearized pGKSD (~6700 bps) and correspondence of linearized pK18*mobsacB* (~5700 bps), b) linearized pGKSUD (~7700 bps), c) double digestion of pGKSUD to excise the insert (~1 kb), L: 1 kb DNA ladder.**

The constructs were transformed into *E.coli* S17-1 and conjugated with *R.capsulatus* DSM1710. Single recombinants were selected as colonies formed on BP plates containing 25 µg/mL kanamycin.

Double recombinants were obtained as described in Section 2.6. Colony PCR was performed for the colonies which grew on sucrose plates but not on kanamycin plates. The double recombinants lost their resistance to kanamycin after the second homologous recombination, since the entire plasmid was excised from genomic DNA of the mutants. The deletion mutation in the gene was confirmed by observation of the short amplicon (400 bps) obtained from colony PCR (Figure 3), as 626 bps long amplicon was routinely obtained from WT cells of the same PCR. The final confirmation step was the sequencing the genomic DNA of the mutant candidate, which also confirmed the deletion site in *glnB* gene.

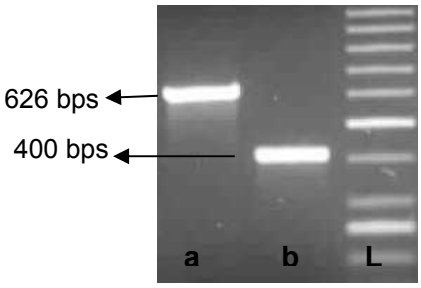

Figure 3: Colony PCR result for wild type *R.capsulatus* (a), *R.capsulatus glnB*⁻ mutant (b) and 50 bp DNA ladder (L).

The study to obtain the double mutant of *glnB* and *glnK* is currently continuing. After the double *glnB*⁻*glnK*⁻ mutant is obtained; the strain will be tested for hydrogen production using different ammonium containing media.

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