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Comparative genomic analysis of two heat-resistant *Rhodobacter capsulatus* mutants with different hydrogen production levels reveals mutations related to hydrogen production

Abdulmecit Gökçe [a,b,c], *Yavuz Öztürk* [d,1], *Zeynep Petek Çakar* [a,b,*]

[a] *Department of Molecular Biology and Genetics, Faculty of Science & Letters, Istanbul Technical University, 34469 Maslak, Istanbul, Turkey*

[b] *Istanbul Technical University, Dr. Orhan Öcalgiray Molecular Biology, Biotechnology & Genetics Research Center, ITU-MOBGAM, 34469 Maslak, Istanbul, Turkey*

[c] *Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Bahcesehir University, 34353 Besiktas, Istanbul, Turkey*

[d] *TÜBİTAK Research Institute for Genetic Engineering and Biotechnology, Kocaeli 41470, Turkey*

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ABSTRACT

To overcome efficiency losses due to overheating in outdoor photobioreactors for microbial hydrogen production, heat-resistant microorganisms are desirable. In this study, the comparative whole genome sequencing analyses of two previously obtained *Rhodobacter capsulatus* heat-resistant mutants; A52 and B41, with modified hydrogen production capacities, have been performed to identify mutations related to hydrogen production. In comparison with the reference strain DSM1710, the genomes of the mutants A52 and B41 contained 2137 and 2253 mutations, respectively. In the mutant B41 genome, mutations were characterized within the *nifD*, *nifJ*, *glnD*, *nifB1*, *ccpA*, *hupD*, *dmsA*, and *cbbR1* genes. On the other hand; in the A52 mutant, mutations were characterized in the *nifB2*, *rnfF*, *nifJ*, *cbbO*, *anfH*, *amt*, *moeA*, and *hupD* genes. The effects of nitrogen metabolism and redox-related mutations were tested by quantitative reverse transcription PCR. The most promising mutation was found on the *glnD* gene of B41 strain with higher hydrogen production capacity.

Abbreviations: Hydrogen, H_2; Nitrogen, N; Quantitative reverse transcription polymerase chain reaction, qRT-PCR; Next generation sequencing, NGS; Calvin–Benson–Bassham cycle, CBB cycle; 1,5-bisphosphate carboxylase/oxygenase, RubisCO; Dimethyl sulfoxide, DMSO; Dimethyl sulfoxide reductase, DMSOR; Purple nonsulfur bacteria, PNS bacteria; Nitrogen regulation system, Ntr; The uridylyl transferase/uridylyl-removing enzyme, GlnD; Uptake hydrogenase, Hup.

* *Corresponding author*. Department of Molecular Biology and Genetics, Faculty of Science & Letters, Istanbul Technical University, 34469 Maslak, Istanbul, Turkey.
E-mail address: cakarp@itu.edu.tr (Z.P. Çakar).
[1] Present address: 1855 sk., No:10, D:5, 41180, Beşevler, Gebze, Kocaeli, Turkey.

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Introduction

Unhindered population growth, the impending shortage of energy resources and environmental problems emerging from the use of fossil fuels are leading the scientists to search for alternative energy sources, including wind, solar and biomass energy, and hydrogen (H_2) production [1]. Majority of the H_2, nowadays, is being produced through physicochemical methods that involve the use of conventional energy sources such as (i) steam reforming of natural gas, (ii) partial oxidation of hydrocarbons, and (iii) coal gasification; or through the use of renewable energy sources like (i) solar photovoltaic power for direct conversion, (ii) wind power, and (iii) hydropower [2]. However, the most promising way of obtaining renewable energy is the production of H_2 using photosynthetic microorganisms such as algae or purple nonsulfur (PNS) bacteria [3]; since the generation of H_2 through photosynthesis has several advantages such as zero emission of greenhouse gases and other environmental pollutants, and production at ambient temperatures with minimal energy consumption [4,5].

Rhodobacter capsulatus is a PNS photosynthetic alphaproteobacterium which is known for its metabolic plasticity enabling it to thrive in disparate ecological niches [6,7]. This feature of *R. capsulatus* makes it an ideal model for the study of respiratory, photosynthetic and chemolithotrophic growth modes [6–8]. For the last two decades, *R. capsulatus* particularly stands out for its ability to produce H_2 under nitrogen limiting conditions by using simple organic acids, such as acetic acid, lactic acid, malate, and butyrate as the carbon source [7–9].

The major route for H_2 production in *R. capsulatus* is the nitrogen fixation [6]. This process is catalyzed by the nitrogenase enzyme, by which H_2 is produced as a byproduct to enable cells to synthesize ammonia from nitrogen gas [10,11].

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad (1)$$

Since the nitrogen fixation process is highly energy-demanding, nitrogenase expression is strictly regulated at three different levels (Fig. 1) influenced by environmental factors such as availability of ammonium, light and the presence of oxygen [12].

At the first level of regulation (Fig. 1A), ammonium availability directs the Ntr system which controls the transcription of *nifA* and *anfA* genes, activators of all nitrogenase encoding genes [12]. Ntr system comprises five proteins: GlnD, the uridylyl transferase/uridylyl-removing enzyme; GlnB, the signal transduction protein (PII); GlnK, a PII paralogue; and two component response regulators NtrB and NtrC. Under nitrogen-limited conditions, PII is uridylylated by GlnD which can sense the nitrogen status of the cell by glutamine/2-ketoglutarate ratio [13]. When uridylylated, PII can no longer interact with NtrB, allowing NtrB to phosphorylate the NtrC response regulator. Phosphorylated NtrC (NtrC-P) activates the expression of its target genes including *nifA*, *anfA*, *glnK* and ammonium transporters *amtB* and *amtY*. At the second level (Fig. 1B), post-translational activity control of NifA is regulated by GlnB and GlnK [11]. In the presence of ammonium, GlnB and GlnK regulate the activity of NifA to repress the expression of *nif* genes [12]. At the third level (Fig. 1C), GlnB, GlnK and ammonium transporters AmtB and AmtY regulate the nitrogenase reductase (NifH and AnfH) activity through reversible ADP-ribosylation in response to ammonium availability or light intensity. In the presence of ammonium, the nitrogenase reductase components of two alternative nitrogenases are ADP-ribosylated, and nitrogenase activity is reduced substantially [10].

Although the H_2 production in *R. capsulatus* is the product of nitrogenase driven N_2 assimilation, the rate of H_2 production is not just affected by this mechanism but also affected by other redox balancing systems which are competing for the electrons. The initial mechanism which maintains the intracellular redox balance in PNS bacteria is the CBB cycle when cells are cultured photoheterotrophically. However, specific conditions such as N_2 limitation and the presence of DMSO as an electron acceptor drives the alternative redox balancing systems; the nitrogenase and DMSOR, respectively [14]. It has been reported that the CBB-deficient mutant strains of *R. capsulatus* derepress the synthesis of the dinitrogenase system, and the H_2 production enhances [15]. However, in the presence of DMSO, the derepression of dinitrogenase protein accumulation or *nifH* promoter activity is severely diminished [15] due to sharing of electrons between nitrogenase and DMSOR systems.

To produce biohydrogen at industrial scale; a number of different photobioreactors have been designed, with differing sources of illumination [16]. Indoor photobioreactors require artificial illumination for H_2 production, while outdoor photobioreactors have the advantage of using the sun as the light source. However, it is impossible to control environmental conditions such as temperature in the outdoor photobioreactors [17]. In order to achieve a sustainable H_2 production in outdoor photobioreactors, two heat-resistant mutant strains (A52 and B41) of *R. capsulatus* were previously developed by our group through a directed evolution approach [16]. According to total H_2 production; A52 strain produces 7% less and B41 strain produces 24% more H_2, compared to the wild-type strain DSM1710 [16] from which both strains were derived. The substrate conversion efficiencies of the mutant strains are 20.16% and 26.73% for A52 and B41 strains, respectively. The hydrogen yield of the A52 and B41 mutant strains are 1.21 and 1.61 mol H_2/mol malate, respectively (Table 1).

In this study, comparative genomic analysis of two heat-resistant mutant strains of *R. capsulatus* (A52 and B41) with different H_2 production levels was conducted in order to determine which mutations affect H_2 production metabolism. In addition to genomic analysis, expression levels of critical genes with mutations were determined, including nitrogen fixation (*nif*) genes, *nifA*, *ntrC*, and *nifH*; RubisCO structural genes *cbbL*, *cbbS* and *cbbO*; PII uridylyl-transferase-encoding gene *glnD*; nitrogenase iron protein-encoding gene *anfH*; hydrogenase maturation protease-encoding *hupD*; molybdopterin biosynthesis protein-encoding *moeA*; ammonium transporter gene *amt*; and dimethyl sulfoxide reductase A subunit-encoding *dmsA* genes. Mutations affecting *cbbR1*, *dmsA*, and *glnD* were proposed as critical mutations which possibly changed the hydrogen production profiles of the mutants.