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Improving the Hydrogen Production Capacity of *Rhodobacter Capsulatus* by Genetically Modifying Redox Balancing Pathways

Yavuz Öztürk, TÜBİTAK Research Institute for Genetic Engineering and Biotechnology, Gebze Kocaeli, Turkey
Abdulmecit Gökçe, Department of Mol. Biology and Genetics, Istanbul Technical University, Turkey
Muazzez Gürgan, **Meral Yücel**, Department of Biology, Middle East Technical University, Turkey

Abstract

In *Rhodobacter capsulatus*, balancing the oxidation-reduction potential (redox-balance) is maintained via a number of inter-dependent regulatory mechanisms that enable these organisms to accommodate divergent growth modes. In order to maintain redox homeostasis, this bacterium possesses regulatory mechanisms functioning as electron sinks affecting the oxidation-reduction state of the ubiquinone pool. Under the photoheterotrophic growth conditions with reduced carbon sources, the excess reducing equivalents are primarily consumed via the reduction of CO_2 through the Calvin–Benson–Bassham (CBB) pathway or by the reduction of protons into hydrogen with the use of dinitrogenase enzyme system. In this study, our aim was to develop strategies to funnel the excess reducing equivalents to nitrogenase-dependent hydrogen production by blocking the carbon-fixation pathway. To realize this purpose, CO_2 fixation was blocked by inactivating the Phosphoribulokinase (PRK) of CBB pathway in wild type (MT1131), uptake-hydrogenase (YO3) and cyt cbb_3 oxidase deficient (YO4) strains. The hydrogen production capacity of newly generated strains deficient in the Calvin-Benson-Bassham pathway were analyzed and compared with wild type strains. The results indicated that, the hydrogen production efficiency and capacity of *R. capsulatus* was further improved by directing the excess reducing equivalents to dinitrogenase-dependent hydrogen production.

1 Introduction

The facultative phototrophic bacterium *Rhodobacter capsulatus* has a number of metabolic pathways to grow under different environmental conditions by performing aerobic respiration, anaerobic respiration, photosynthesis and fermentation [1]. Under photoheterotrophic growth conditions, the oxidation of organic acids (such as malate, acetate and lactate) can result in overreduction of the ubiquinone pool. As cyclic photosynthesis requires oxidized ubiquinone as an electron acceptor, excess reducing equivalents, at the level of the reduced ubiquinone pool are removed by redox-balancing systems [2]. The maintenance of intracellular redox poise was achieved by dissipating reducing equivalents through the CBB cycle, the DMSOR system, or the dinitrogenase system. The CBB pathway, rather than serving as a major means for generating organic carbon under these growth conditions, plays a role in redox

balance of the cell when carbon substrates are oxidized [3]. The dinitrogenase enzyme system, besides its role in nitrogen metabolism, also serves as a redox-balancing system during photoheterotrophic growth under limiting nitrogen sources [4]. Under such conditions, the nitrogenase is activated and the excess reducing equivalents generated by the oxidation of organic acids are consumed by the reduction of protons to molecular hydrogen [5]. Previously, hydrogen production properties of some uptake hydrogenase deleted *R. capsulatus* strains and mutants with genetically modified electron transfer chains were investigated and reported [6, 7]. In this study, we investigate the relationship between the redox balancing system and hydrogen production in various *R. capsulatus* strains deficient in CBB pathway, terminal oxidases and uptake hydrogenase.

2 Materials and Methods

Bacterial Strains and Growth conditions:

The bacterial strains used are described in Table 1. R. *capsulatus* strains were grown on MPYE (magnesium-calcium, peptone, yeast extract) enriched medium [8] and Sistrom's minimal medium A (Med A, containing succinate as carbon and NH_4^+ as nitrogen sources) at 35° C and supplemented with kanamycin, gentamicin and spectinomycin (10, 1, 10 µg per ml, respectively). Ps cultures were incubated under saturating light intensity in anaerobic jars containing H_2 and CO_2 generating gas packs (BBL 270304, Becton Dickenson and Inc). For hydrogen production experiments, the minimal medium of Biebl and Pfennig (BP), supplied with C/N sources (15 mM/2 mM for malate/L-glutamate) without ammonium chloride and yeast extract, was used [9].

Molecular Genetic Techniques

Inactivation of *cbbP* gene*:*

The ∆(*cbbP*::*Spe*) deletion-insertion allele was constructed by replacing the 180 bp *Bam*HI fragment of *cbbP* in plasmid pYO50 with the 2 kb *Bam*HI fragment containing spectinomycin resistance gene, yielding pYO51 (Figure 1). Fragment containing the ∆(*cbbP*::*Spe*) deletion-insertion allele was then cloned in the broad host range plasmid pRK415, yielding pYO52, to transfer the mutant allele to gene transfer agent (GTA) overproducer *R. capsulatus* Y_{262} strain via triparental mating [19]. GTA cross was used to inactivate the chromosomal copy of *cbbP* in *R. capsulatus* MT1131, YO3 (*Hup*$^-$) and YO4 (cyt *cbb*$_3^-$, *Hup*$^-$).

Table 1: *Rhodobacter capsulatus* strains used in this work.

Strain	Genotype	Reference
MT1131	*crtD121*, Wild type, Rif^R	[8]
YO3	*hupSLC*$^-$	[6]
YO4	*hupSLC*$^-$, cyt *cbb*$_3^-$	[6]
YO6	*cbbP*$^-$	This work
YO7	*hupSLC*$^-$, *cbbP*$^-$	This work
YO8	*hupSLC*$^-$, cyt *cbb*$_3^-$, *cbbP*$^-$	This work

3 Results

3.1 Chromosomal inactivation of phosphoribulokinase of *R. capsulatus* strains.

The Phosphoribulokinase deficient (PRK$^-$) derivatives of *R. capsulatus* MT1131 (wild type), YO3 (*hup*$^-$) and YO4 (cyt *cbb*$_3^-$, *hup*$^-$) strains were obtained by gene transfer agent (GTA) crosses yielding the strains YO6, YO7 and YO8, respectively (Table 1). Chromosomal inactivation of *cbbP* in these strains was confirmed by PCR amplification of the Δ*cbbP*::Spe allele and by photosynthetic phenotypes in a NH_4^+ containing minimal medium (Figure 1).

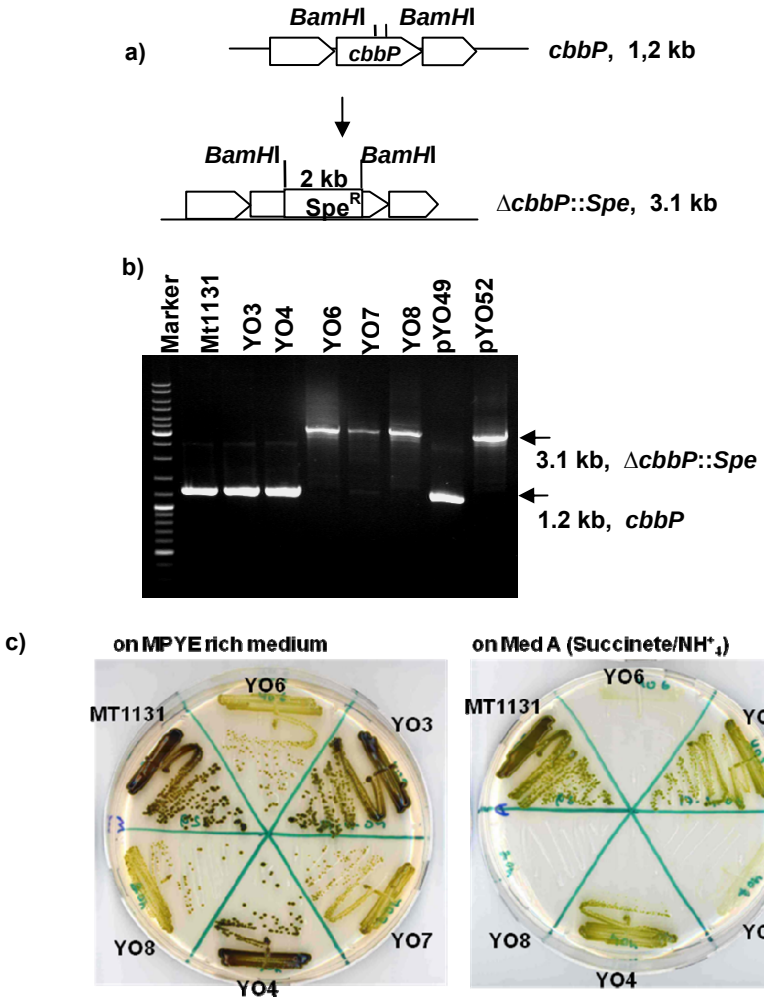

Figure 1: **The Δ*cbbP*::Spe deletion-insertion allele was constructed by replacing the 180 bp *BamH*I fragment of *cbbP* by a 2 kb Spectinomycin cassette (a). *cbbP* deletion was confirmed by PCR amplification of *cbbP* and Δ*cbbP*::Spe insertion-deletion allele from chromosomal DNAs of wild type and mutant *R. capsulatus* strains. pYO49 and pYO52 are the plasmids carrying *cbbP* and Δ*cbbP*::Spe in pBSII respectively (b). Photosynthetic growth properties of various *R. capsulatus* strains on MPYE rich medium (c-left plate) and on minimal medium MedA (succinete/NH^+_4) (c-right plate). The growth of PRK$^-$ derivatives deficient in CBB pathway are very slow on Nitrogenase repressing medium MedA.**

3.2 Hydrogen production by PRK$^-$ strains of R. capsulatus.

Hydrogen production profiles of the newly generated PRK$^-$ strains YO6, YO7 and YO8 were investigated and compared to their respective PRK$^+$ parents. The data obtained demonstrated that the total hydrogen production and substrate conversion efficiency of YO7 and YO8 strains increased as shown in Figure 2, and Table 2. For example, the total hydrogen production and substrate conversation efficiency of YO7 (*Hup*$^-$, PRK$^-$) were 1.15 and 57% respectively. The total hydrogen production was increased 1. 6 fold as compared with the wild type MT1131 and 1.1 fold as compared with the YO3. Likewise, the total hydrogen production and substrate conversation efficiency of YO8 (cyt cbb_3^-, *Hup*$^-$, PRK$^-$) were increased 1.2 fold and 1.1 when comparing the wild type MT1131 and YO4 respectively. The data obtained demonstrated that the inactivating the CBB pathway especially in YO3 and YO4 strains further improved the hydrogen production capacity of the *R. capsulatus* strains. Moreover, under the photoheterotrophic growth condition in minimal medium with ammonium as an nitrogen source, the growth of all PRK$^-$ strains were shown to be impaired in their ability to grow photoheterotrophically due to the lack of a functional CBB cycle and repression of the dinitrogenase system (Figure 1-c). However they gave Ps$^+$ revertants frequently in this medium and several spontaneous revertant variants of PRK$^-$ strains that have become photoheterotrophically competent were isolated. These strains were shown to obviate normal ammonia control and derepress synthesis of the dinitrogenase enzyme complex for the dissipation of excess reducing equivalents and generation of H_2 gas via proton reduction.

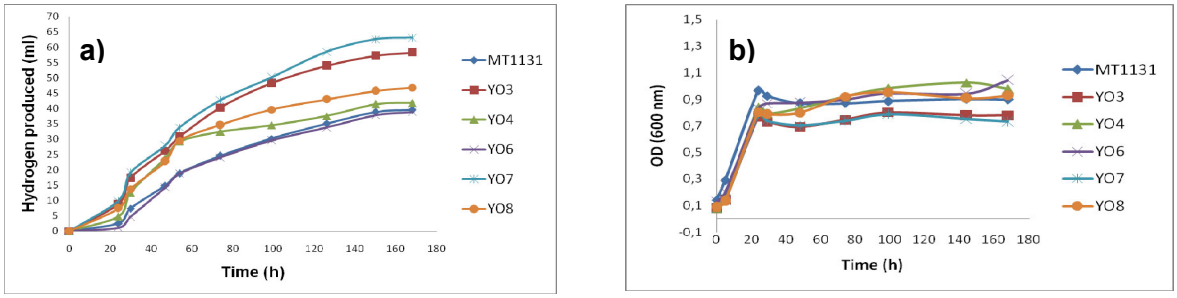

Figure 2: Hydrogen production (a) and phototrophic growth (b) of PRK$^-$ mutants and their respective PRK$^+$ parent strains.

Table 2: Hydrogen production of wild type and different mutants of *R. capsulatus*. The data are means of three different determinations.

strains	Total Hydrogen (ml/ml culture)	Substrate conversion efficiency (%)
Mt1131	0,72	36
YO3 (*Hup*$^-$)	1,06	52
YO4 (cyt *cbb*$_3^-$, *Hup*$^-$)	0,76	38
YO6 (*cbbP*$^-$)	0,71	35
YO7 (*Hup*$^-$, *cbbP*$^-$)	1,15	57
YO8 (cyt *cbb*$_3^-$, *Hup*$^-$, *cbbP*$^-$)	0,85	42

4 Conclusion

Rhodobacter capsulatus produces molecular hydrogen under the photoheterotrophic growth condition with reduced carbon sources (organic acids). Under the this condition, ubiquinol poll is over reduced and excess reducing equivalents are primarily consumed via the reduction of CO_2 through the Calvin–Benson–Bassham (CBB) pathway, the DMSOR system or by the reduction of protons into H_2 gas with the use of dinitrogenase enzyme system to maintain a balanced intracellular oxidation-reduction potential. The relationship between the redox balancing system and the hydrogen metabolism was analyzed by using various mutant strains deficient in terminal oxidases, uptake hydrogenase, Phosphoribulokinase and in their various combinations. The results obtained showed that in the absence of the functional CBB pathway, the excess reducing equivalents were dissipated only through the dinitrogenase system in the form of more hydrogen. Besides, the preliminary data of some isolated revertant of PRK$^-$ strains indicated that hydrogen gas production of these strains were further enhanced by derepression of dinitrogenase system removing the redox poising by H_2 production.

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Nomenclature:

CBB pathway: Calvin–Benson–Bassham reductive pentose phosphate pathway; PRK: Phosphoribulokinase; Cyt *cbb*$_3$ ox: Cytochrome *cbb*$_3$ oxidase; *Hup*: Uptake hydrogenase

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